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ATROPHY OF THE PROXIMAL SEGMENT AND REINNERVATION OF MUSCLE

by

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ABSTRACT

Part I

The atrophy of cutaneous (sural) and muscle (medial gastrocnemius) nerves proximal to a ligation was studied in cats for periods up to 9 months, using light and electron microscopy, conduction velocity measurements and computer simulations of action potential conduction. Perimeters of axons and fibres (axon + myelin) were measured and cross-sectional areas and diameters of "equivalent circles" calculated. As atrophy proceeded, nerve fibres became increasingly noncircular. A linear relationship was found between axon diameter and fibre diameter, but decreasing slope as atrophy continued indicated that the axon cross-sectional area decreased relatively more than the total fibre area. Reduction in conduction velocity correlated more closely with reduction in axon diameter than fibre (axon + myelin) diameter.

The ratio of axon perimeter to myelin perimeter remained constant at or near the optimal value of 0.6 for conduction in all groups of fibres at all periods of atrophy studied. This suggests that the number of turns of myelin and the length of each turn remained unchanged during peripheral nerve atrophy. A simple geometrical model explains how this can occur. The Frankenhaeuser-Huxley equations for conduction in myelinated nerve fibres predict changes in conduction velocity similar to those observed if the axons atrophy without changes in myelin.

Part II

The physiological properties of the fast lateral gastrocnemius and slow soleus muscles and their motor unit populations were studied after reinnervation by their own nerve in control and experimental animals. Histochemical stains for oxidative, glycolytic and myosin ATPase enzyme activity were used to identify fibre types in control and reinnervated muscles and motor unit and histochemical properties compared.

Reinnervated muscles recovered a good proportion of their weight, number of fibres and force. Fibre types became almost completely "type-grouped". Both reinnervated muscles contained the same proportion of slow and fast motor units. Slow and fast muscle fibres were not preferentially reinnervated by slow and fast nerve fibres respectively. The fast muscle became slower and the slow muscle became faster. Alterations in the times and rates of development and relaxation of tension of whole muscle twitch and tetanic contractions could be explained by the altered motor unit population in the reinnervated muscles.

Motor units were stimulated by isolation of single motor axons and their contractile properties studied as for whole muscles. Motor units were classified as FF (fast, fatiguable), FI (fast intermediate), FR (fast, fatigue-resistant) and S (slow) according to their contraction speed and resistance to fatigue. Contractile properties measured to determine motor unit characteristics

were not significantly different from controls in each type of reinnervated motor unit in both formerly fast and slow muscles after reinnervation.

The normal relationships between the size of the motor unit as indicated by twitch and tetanic force and the innervation ratio (the number of muscle fibres per motor unit), the contraction time, fatigue resistance, and the type of motor unit were found for the fast motor units in reinnervated muscle although these motor units remained within a smaller range of sizes than in control muscle. Slow motor units were larger than controls in both reinnervated muscles and the average twitch forces were not significantly different from each other or from reinnervated FR motor units in either muscle. Specific tension (per fibre) was decreased in some motor units after reinnervation. Fibre counts may have underestimated the actual number of fibres by 20% in control muscle and by a larger percentage in atrophied muscle.

Proportions of motor units and corresponding histochemical fibre types were well correlated in the LG control and reinnervated muscles and in control soleus. Reinnervated soleus contained a high proportion of oxidative fibres with histochemically slow myosin ATPase while having a much lower proportion of slow motor units and a faster contraction time than control soleus. The oxidative properties of soleus muscle fibres are resistant to alteration by the nerve.

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I am indebted to Dr. Tessa Gordon and Dr. Peter Murphy who collaborated on the research presented in the second chapter.

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1. PERIPHERAL NERVE INJURY: THE EFFECTS OF AXOTOMY

1.1 INTRODUCTION

The sequence of events following axotomy includes: Wallerian degeneration of the distal segment, axonal atrophy of the proximal stump and chromatolysis of the cell body followed usually by the formation of sprouts and their regrowth towards the periphery and reinnervation of the target organ. Successful reinnervation of the appropriate or original target requires some degree of specificity of innervation and raises the question of the trophic influences acting between neuron and target. This study[1] examines two phases of the events occurring subsequent to axotomy:

1. the atrophy of the proximal portion of the axon when reinnervation is prevented and
2. the specificity of a motor neuron for its target muscle on reinnervation of the muscle.

1.1.0.1 The Reaction of the Distal Stump

The degeneration of the distal stump is named after Waller who first described the process in 1850. More recent findings describe an accumulation of organelles, disruption of the axonal cytoskeleton, and swelling and disruption of the axon and the myelin. The Schwann cells are stimulated to proliferate and phagocytize the axon and myelin debris. Finally there remains a collapsed endoneurial tube filled with Schwann cells which can serve as a pathway for the growth of regenerating nerve sprouts.

---

[1] Results of the experiment presented in this chapter were published in "The relationship between axon, myelin and conduction velocity during atrophy of mammalian peripheral nerves" *Brain Research, 1983*, 259, 41-56, by M.J. Gillespie and R.B. Stein. The work is included in this thesis with the kind permission of the co-author, Dr. Stein.

1.1.0.2 The Reaction of the Cell Body

The typical reaction of the majority of cell bodies, first described by Nissl in 1894 is swelling of the perikaryon, movement of the nucleus to an eccentric position, where it is flattened against the membrane at the pole of the cell opposite the axon hillock, and the dispersion and disappearance of the basophilic Nissl substance. The reaction can be detected within 24 hours (Matthews & Raisman 1972) and is well developed by 2-3 days. Recent investigations with the electron microscope have confirmed the original observations and provided greater detail. Loosening of the structure of the basophilic substance, degranulation and dispersal of the rough endoplasmic reticulum and disaggregation of the cytoplasmic ribosomes are the ultrastructural correlates of the chromatolytic reaction (Smith 1961; Mackey et al 1964; Zelena 1971; Matthews & Raisman 1972; Barron & Dentinger 1979). The detachment of the ribosomes from the rough endoplasmic reticulum accompanies an increase in the production of RNA and protein (Watson 1970) as the metabolism of the neuron switches from the production of transmitter-associated molecules to anabolic regenerative processes (Harkonen & Kaufman 1973; 1974). Hypertrophy and peripheral displacement of the Golgi apparatus have been reported (Bodian 1964; Lieberman 1969; Barron & Dentinger 1979). Swelling and increased numbers of mitochondria (Hartmann 1954; Hudson et al 1961), indentation of the nuclear membrane (Hartmann 1954; Bodian 1964), the appearance of cytoplasmic organelles (Zelena 1971; Matthews & Raisman 1972) and increased numbers of neurofilaments (Zelena 1971; Barron & Dentinger 1979) are other characteristic features of the reaction.

Alteration of the neuronal membrane and hyperplasia of glial cells (Kirkpatrick 1968; Chen 1978) lead to degeneration and retraction of the cell's afferent synapses (Kerns & Hinsman 1973; Sumner 1975) and the insertion of glial processes between the boutons and the cell membrane (Kerns & Hinsman 1973; Mendell, Munson & Scott 1976). Altered membrane properties also result in reduced synaptic efficacy accompanied by reduction in the size and rate of rise of the monosynaptic excitatory postsynaptic potential (Downman, Eccles & McIntyre 1953; Eccles Krnjevic & Miledi 1959; Kiraly & Krnjevic 1959; Kuno & Linas 1970).

The chromatolytic reaction is usually maximal between seven and fourteen days (Zelena 1971; Matthews & Raisman 1972). Thereafter fewer chromatolytic cells are seen (Barron & Dentinger 1969; Zelena 1971). The duration and severity of the chromatolytic reaction is influenced by a number of factors. Cells of foetal, new-born and early post-natal animals are particularly susceptible to injury and most often die following axotomy (Aldoskogius & Risling 1981) while adult cells show variable degrees of recovery. The proximity of the lesion to the cell body also influences the severity of the reaction, with more rapid onset and more severe and prolonged reaction occurring when the lesion is close to the perikaryon. More extensive chromatolysis is thought to be an indicator or precursor of cell death (Barr & Hamilton 1948). The nature of the lesion also has an effect on the extent of cell reaction and neuronal degeneration. Crush injuries generally allow for full restoration of function while recovery is less complete following nerve section. Species differences in cell reaction of both neurons and satellite cells have also been found (reviewed by Liebermann 1974).

The chromatolytic reaction described above is most typical of vertebrate 'peripheral' neurons. Matthews & Raisman (1972) reported that the majority (85%) of the motor neurons show signs of reaction after sciatic nerve section in the rat. Ranson (1906) cited reports of variable degrees of reaction and variable extent of cell loss, particularly with respect to sensory versus motor cells; i.e. the response of ventral horn cells was variable, but there was a consistent loss of half the spinal ganglion cells. Smith et al (1960) reported that large cells were more often involved than small cells while Risling et al (1980) found large and small cells to be equally affected. Some investigations report cell death of one third to one half of all cells (Aldoskogius & Risling 1981) or there may be recovery from chromatolysis with long term atrophy of the cells (Barr & Hamilton 1948).

1.1.0.3 The Reaction of the Proximal Segment of the Axon

Cajal (1928) described the development of a necrotic segment at the end of an interrupted axon. The axoplasm becomes granular for up to 3 nodes (Blumcke, Nierdorf & Rodej 1966; Zelena, Lubinska & Guttman 1968), the granular region continuing to extend over a period of 29 hours or more (Causey & Palmer 1952; Lubinska 1961; Morgan, Hughes & Engel 1968; Morris, Hudson & Weddell 1972). Organelles (Zelena, Lubinska & Gutmann 1968) enzymes, (Morgan, Hughes & Engel 1968) lipids, (Aldoskogius 1978) dense lamellar bodies, vesicles, autolytic bodies and tubular structures (Blumcke, Neirdorf & Rodej 1966; Morris, Hudson & Weddell 1972c) are seen to accumulate proximal to the zone of necrosis and the neurofilaments are disrupted (Zelena 1971; Schlaeffer & Micko 1978). There is demyelination of this necrotic segment (Causey & Palmer 1952; Lubinska 1961; Blumcke, Nierdorf & Rodej 1966), but not necessarily demyelination of the rest of the proximal segment (Lampert 1967). Morris, Hudson & Weddell (1972a) described withdrawal of Schwann cells of this segment and phagocytosis of myelin by them. Schwann cells containing myelin debris were seen for up to 10 days following the injury, after which there was a gradual disappearance of both. Protein synthesis in the axon has been reported (Tobias & Koenig 1975; Benech et al 1982).

After a latent period during the initial reaction of between 2 and 24 hours the distal portion of the axon develops a terminal swelling or "club of growth" (Ramon y Cajal 1928; Blumcke, Nierdorf & Rodej 1966) and within the first six days a number of sprouts grow from the ends of the bulb and from adjacent regions of the axon. These sprouts grow towards the injured region (Cajal 1928), slowly penetrate the scar and then grow towards the periphery at a fairly constant rate of 2-3 mm per day (Cajal 1928; Grafstein & McQuarrie 1978). During their progress they may divide, be diverted or even turned back (Scadding & Thomas 1983). Successful sprouts are seen to enter the old endoneurial tubes and grow down them towards their original target.

1.1.0.4 Atrophy of the Axon When Reinnervation is Prevented

When reinnervation of the periphery is prevented, the axons of the proximal segment above the injured zone may survive indefinitely, undergoing gradual atrophy

(Gutmann & Holubar 1951; Cragg & Thomas 1961; Aitken & Thomas 1962). Several studies have reported greater effects of axotomy on sensory fibres than on motor fibres (Carlson et al 1979; Hoffer, Stein & Gordon 1979; Risling et al 1980; Milner & Stein 1981). Sanders (1948), Cragg & Thomas (1961) and Aitken & Thomas (1962) in their light microscope observations reported that the diameter of the axon was reduced more than the total diameter of the fibre (the axon plus the myelin sheath). Sanders (1948) reported that myelin thickness was increased while Gutmann & Holubar (1951) found it decreased. More recent studies with the electron microscope have not definitively settled the question (Spencer & Thomas 1970; Carlson, Lais & Dyck 1979). It is clear that as a nerve fibre atrophies, the myelin occupies an increasing fraction of the cross-sectional area, but it is uncertain whether this represents an absolute increase in myelin thickness or a relative increase when compared to the reduced size of the axon.

1.1.0.5 Conduction Velocity of Atrophied Nerves

There is a decrease in conduction velocity proximal to a peripheral nerve lesion which accompanies the atrophy of the fibres (Cragg & Thomas 1961; Kuno et al 1974; Mendell et al 1976). The loss of fibre diameter and reduced conduction velocity can reach a plateau and the fibres can remain atrophied indefinitely without further deterioration (Guttman & Sanders 1942; Cragg &

Thomas 1961; Czeh, Kudo & Kuno 1977; Milner & Stein 1981). The nerve continues to be able to generate action potentials (De Luca & Gilmore 1976; Hoffer, Gordon & Stein 1979). The reduction in the charge generated at the dorsal and ventral roots and the reduction in conduction velocity were both seen to be greater in large sensory fibres than in motor fibres (Hoffer, Stein & Gordon 1979; Milner & Stein 1981). Milner & Stein (1981) cited preliminary observations that the decline in conduction velocity in atrophic nerves appeared to be much greater than the decline in fibre diameter measured histologically. Conduction velocity has been correlated with outside fibre diameter in normal myelinated fibres (Hursh 1939).

1.1.0.6 Theories of Conduction Velocity

For myelinated fibres a linear relationship between conduction velocity and diameter has been shown by Hursh (1939), Rushton (1951), Frankenhaeuser & Huxley (1964) Boyd (1966) and Buchtal & Rosenfalck (1966). Boyd & Kalu (1979) and Arbuthnott et al (1980) found different linear relationships between conduction velocity and outer fibre diameter for large and small myelinated fibres. Theoretical approaches (Goldman & Albus 1968; Koumarelas 1973) also predicted the dependence of conduction velocity on fibre diameter. These analyses presumed geometrical uniformity of nerve fibres (Waxman 1980) and failed to consider the variation of other fibre parameters with diameter. Investigators who sought to determine the effects of other parameters, singly or in combination, include those who considered myelin thickness, (Sanders 1948; Sunderland & Roche 1958; Freide & Samorajski 1967; Williams, Wendell-Smith 1971), the internodal length (Hursh 1939; Coppin & Jack 1972; Dyck, Lambert et al 1981), nodal properties (Koles & Rasminsky 1972) and the axon diameter (Donaldson & Hoke 1905; Gasser & Grundfest 1939; Rushton 1951; Hodgkin 1964; Friede & Bischhausen 1980). It was predicted by Rushton (1951), Roche (1958), Sunderland & Roche (1958) Frankenhaeuser & Huxley (1964), Smith & Koles (1970) and Moore, Brill, Joyner et al (1978), that the myelin thickness *m* and the thus the related ratio *g* of axon diameter *d* to total fibre diameter *D* would have an optimum value (0.6 for *g*). There is considerable uncertainty about the relationship of axon diameter to total fibre diameter in normal nerves (Huxley & Stampfli 1949; Rushton 1951; Sunderland & Roche 1958; Buchtal & Rosenfalck 1966; Friede & Samorajski 1967), and almost no information for atrophic nerves.

1.2 THE RELATION BETWEEN AXON DIAMETER, MYELIN THICKNESS and CONDUCTION VELOCITY

The experiment to be described was designed to investigate the correlation between the decline in conduction velocity, and histological measurements of atrophied nerves.[2] A sensory (cutaneous) and a mixed (muscle) nerve were cut in the hind limb of cats and allowed to atrophy for up to 273 days. At regular intervals of approximately 20 days the conduction velocity was measured for both motor and sensory fibres. At the conclusion of the acute experiment, nerve samples were fixed for measurement of diameter. Photomicrographs taken with light and electron microscopes were used to measure axon perimeter and total fibre perimeter, and from these values the equivalent axon and total fibre (axon plus myelin) cross-sectional areas were calculated. ("equivalent" is used to mean the value for a circle having the same perimeter). Equivalent axon and total fibre diameters were likewise calculated and from these the myelin thickness was determined. The relationship of conduction velocity to the above parameters was also calculated using the Frankenhaeuser and Huxley equations. Computer simulations (Smith & Koles 1970; Koles & Rasminsky 1972) have been used to compare the validity of the findings with the theoretical predictions.

1.2.1 Methods

The sural (Su) nerve and the medial gastrocnemius (MG) nerve were cut in one hind limb of 12 cats. The cut and ligated nerves were sewn to a small silastic pad which was placed between the nerve and its former target organ to prevent reinnervation. At intervals of approximately 20 days from 29 to 273 days after nerve section the compound action potentials generated by the cut nerves were studied in acute experiments and the conduction velocity distributions were determined as described by Milner & Stein (1981) and Milner, Stein et al (1981). The unoperated contralateral nerves were used as controls.

---

[2] Much of the material presented in this section was published in *Brain Research* 259 (1983), pp. 41-56.

1.2.1.1 Histology

Light Microscopy

Upon completion of the physiological studies, samples 7 to 15 mm in length of each experimental and control nerve were taken about 10 mm proximal to the cut end or to the recording site. Nerves containing a greatly increased proportion of small myelinated fibres were excluded as this indicates sprouts growing centrally (Scadding & Thomas 1983). Several experimental nerves were also sampled 2-3 cm proximal to the cut as an additional control and of these only those nerves containing the same number of myelinated axons both proximally and distally were included. The nerves were fixed in 3% gluteraldehyde in 0.1M phosphate buffer for 2-6 hours, post-fixed in osmium tetroxide for 1 hour, dried in ascending alcohols and embedded in Araldite. 1 µm thick sections were stained with 1% p-phenylenediamine (aequeous) and photographed with phase-contrast microscopy using a green filter to provide monochromatic illumination. Photographs were printed at 1000x magnification and the diameter of all myelinated fibres in each nerve was measured by fitting with circles of standard sizes. Histograms of fibre size distribution were prepared and the cumulative percentage of fibres present at or below a given total diameter was plotted against $\log_{10}$ fibre diameter.

Electron Microscopy

Electron micrographs of ultra-thin (silver-gray) sections of nerves were printed at a magnification of 9240 times. 50 to 150 fibres per nerve were measured by tracing the inner (axon) perimeter s , and the outer (myelin or total fibre) perimeter S, using a cursor with a cross-hair. The rectangular coordinates of the position of the cross-hair during the tracing were recorded using a microcomputer (LSI-11; Digital Equipment Corp.) and from these values the diameters of circles having equivalent perimeters ($d\ eq$ and $D\ eq$) and the areas (a and A) were calculated.

From these values, other parameters were calculated as follows:

g= the ratio of inner perimeter (s) to outer perimeter (S) (s/S) (Arbuthbott et al 1980). The same letter is often used for the ratio of inner to outer diameter (($d\ eq/D\ eq$), but the two ratios are only the same for a circle and are affected quite differently as nerves atrophy (see Appendix).

m = myelin thickness, calculated from the perimeters. $m = (S - s)/2\pi$. This simple formula turns out to be remarkably accurate (see page 28 and the Appendix) and far less time consuming than trying to measure the thickness directly at a number of points around the fibre or counting the turns of myelin.

ϕ= circularity of the axon, calculated as the axon cross-sectional area (a) divided by the area of a circle having the same perimeter.

$\phi=4\pi\ a/s^2$ (Arbuthbott et al 1980).

For a circle ϕ = 1 and for a slit with zero area ϕ= 0. Thus ϕ provides a convenient normalized measure of circularity.

Φ = circularity of fibre (axon + myelin), calculated as above from the outer measurements

$\Phi = 4\pi\ A/S^2$ where (A) is the cross-sectional area of the fibre.

1.2.1.2 Computed Action Potentials

Action potentials were computed from the Frankenhaeuser-Huxley equations for a frog node, using the cable equations for the internodal regions, as first described by Goldman & Albus (1968). A FORTRAN program modified from Smith & Koles (1970) and Koumarelas (1973) to run on a PDP 11-34 computer was used to calculate action potentials for ten nodes for a fibre at 20°C with an outer diameter of 15 µm and an inner diameter of 10.5 µm. For a circular fibre the ratio $g = d/D = 0.7$. If, during atrophy, there were no changes in the properties of the axon membrane or the myelin or Schwann-cell membrane, the only change would be in the axoplasmic resistance per unit length of cable. During atrophy the axon area *a* will decrease as the axon becomes noncircular, as will the diameter *d* eq of a circle of equivalent size. The axoplasmic resistance Ra will therefore increase, while all other parameters of the equation remain constant. An 8-fold increase in the axoplasmic resistance Ra, corresponding to a 2.8-fold decrease in the equivalent axon diameter was studied. With the higher resistance the stimulus at the first node was adjusted to ensure that an action potential was propagated. In computing conduction velocity the first node was ignored because of possible stimulus artifacts, and the last node was ignored because of the termination of the cable in a short-circuit. The conduction velocity was determined as the time for the half-maximum point on the rising edge of the action potential to propagate over the intermediate nodes (internodal distance = 1.38 mm).

1.2.2 Results

1.2.2.1 Histology

Transverse sections of fixed nerves exhibited the criteria of good fixation outlined by Morris,Hudson & Weddell (1972b). "The myelin rings followed smooth curves without sharp angles and fibres were not packed together with high density, such that the distortion of fibre shape occurred. There was negligible splitting of the myelin lamellae in small and medium sized nerve fibres, and only mild splitting in some of the large fibres, even when the profiles of these were highly crenated". Figure 1.1 is an electron micrograph of part of the cross-section of an MG nerve after 228 days atrophy which demonstrates good fixation of the tissues (magnification x4620). The axons are not compressed or crowded together, the myelin is intact and the axon has not retracted from the inner perimeter of the myelin sheath but continues to fill the space enclosed by it. Intact organelles can be seen in the axoplasm and the Schwann cell cytoplasm.

Cross-sections of the cat cutaneous (sural) and muscle (medial gastrocnemius) nerves are shown in figure 1.2. Sural control is on the upper left and MG control is on the upper right. An experimental sural nerve 253 days after axotomy and a medial gastrocnemius nerve 228 days after axotomy are shown below the controls on the left and right respectively. The majority of the axons in the experimental nerves showed altered profiles compared to the control nerves. Fibres became progressively less round as the time after ligation increased. The myelin sheaths became wavy and involuted in profile but remained intact. The sheaths collapsed into the axonal space as the axons shrank in volume and cross-sectional area.

Figure 1.1 The electron micrograph of a sural nerve cross-section illustrates that the preparations meet the criteria for good fixation of the tissue (Morris, Hudson and Weddell 1972b). The myelin lamellae remain intact and have not separated and the axoplasm has not retracted from the inner perimeter of the myelin sheath. Fibre shapes are not distorted as a result of compression of the tissue. Intact organelles can be seen in the axoplasm and the Schwann cell cytoplasm. (Magnification x4620).

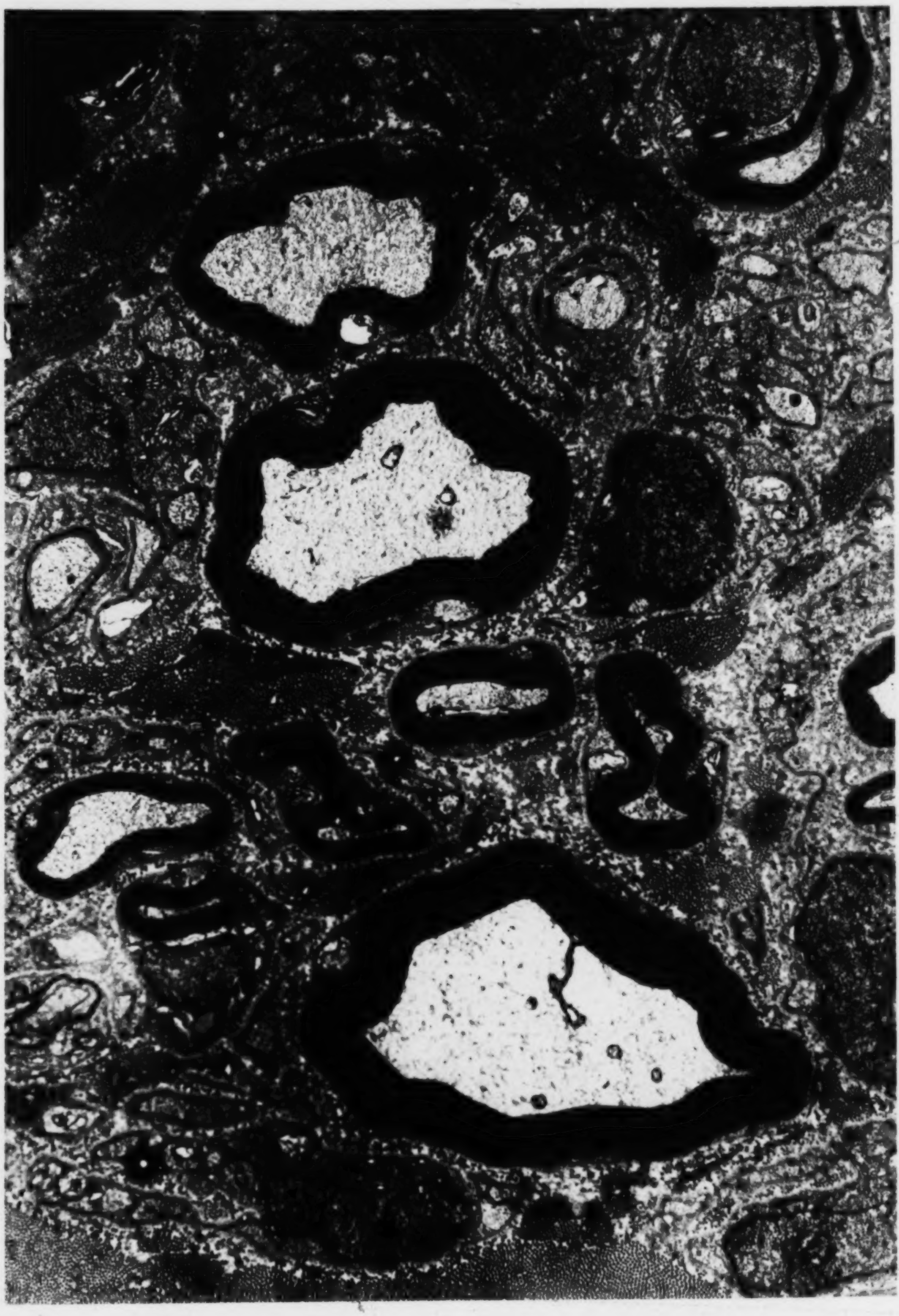
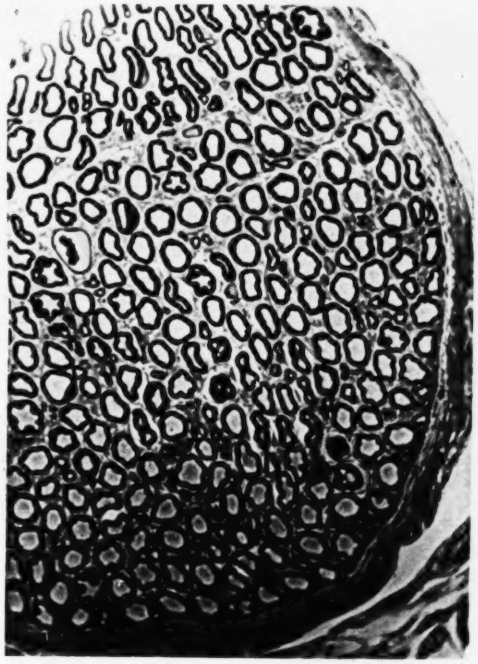
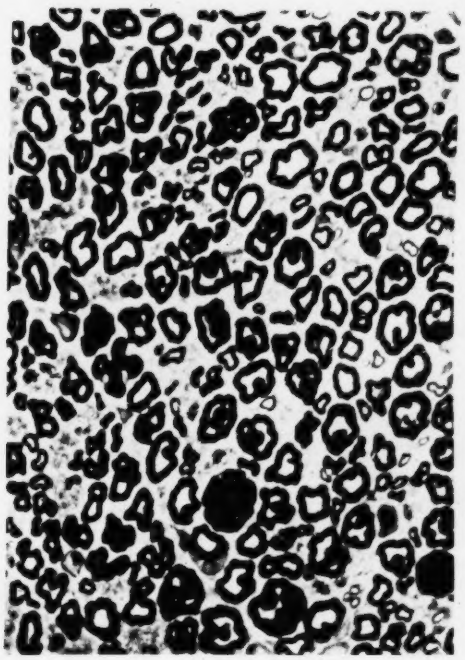
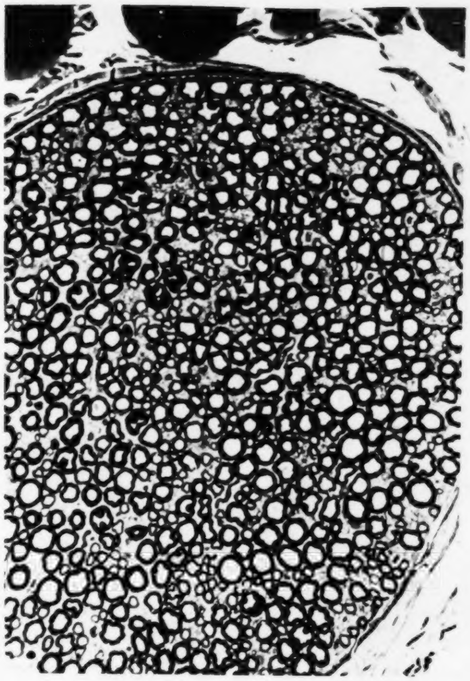
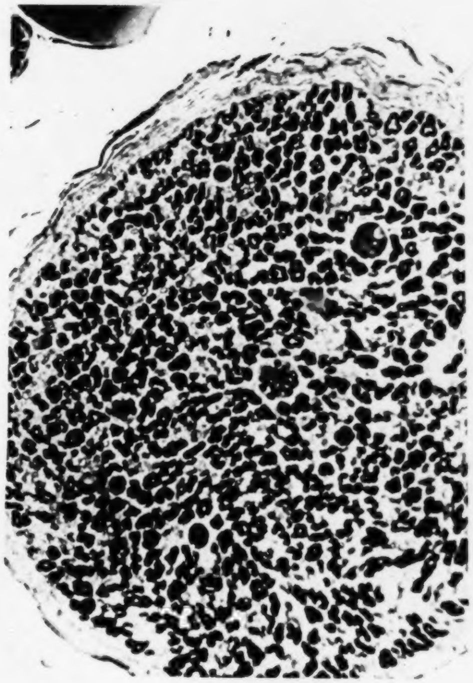


Figure 1.2. Cross-sections of control sural and medial gastrocnemius (MG) nerves (above left and right, respectively) and below, nerves proximal to a ligation after 228 days of atrophy. Note that in these light micrographs many of the fibres in the experimental nerves show little or no clear axoplasm, particularly in the sural nerve. Magnification x225.

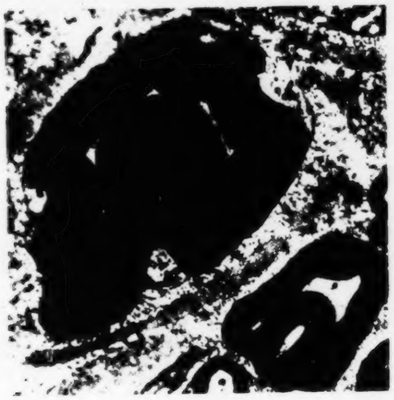
In general, the sural nerve fibres became more oval and flattened and most fibre sheaths resembled completely closed tubes after greater than 200 days atrophy. The electron micrograph in figure 1.3 of individual axons of experimental nerves after more than 200 days (as in figure 1.1) shows that the fibres of the muscle nerve developed regularly indented, wavy profiles and many of the fibres resembled normal para-nodal fibres. Some fibres remained relatively round, perhaps due to the lesser atrophy of motor axons (Hoffer, Stein & Gordon 1979), and few fibres appeared completely collapsed.

Fibre Diameter Histograms

Histograms of fibre diameter (axon + myelin) measured from light micrographs of control sural nerves had a bimodal distribution with peaks at 6μm and 10μm and are shown in figure 1.4 (A). The bimodal distribution was lost in the experimental nerves (figure 1.4 B). The major peak occurred at 4μm and very few fibres were observed greater than 12μm in diameter after greater than 200 days atrophy. Similarly, the control muscle nerves had a bimodal distribution with peaks at about 6μm and 16μm as seen in figure 1.4 (A). After greater than 200 days atrophy only a single peak was observed at 4-6μm diameter and again very few large fibres were observed (figure 1.4 B). Note that the total numbers of nerve fibres was almost identical, even more

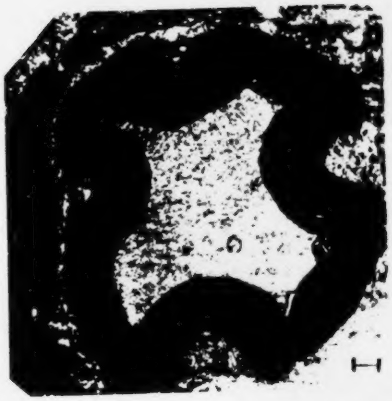
Figure 1.3 Electron micrographs of fibres in the sural and MG nerve over 200 days after the nerves had been ligated. Some fibres had atrophied relatively little (MG) whereas in others the axoplasm had almost disappeared (large sural fibre). The scale bar shown on the right is 1μm.

SURAL NERVE



228 days

MG NERVE



273 days

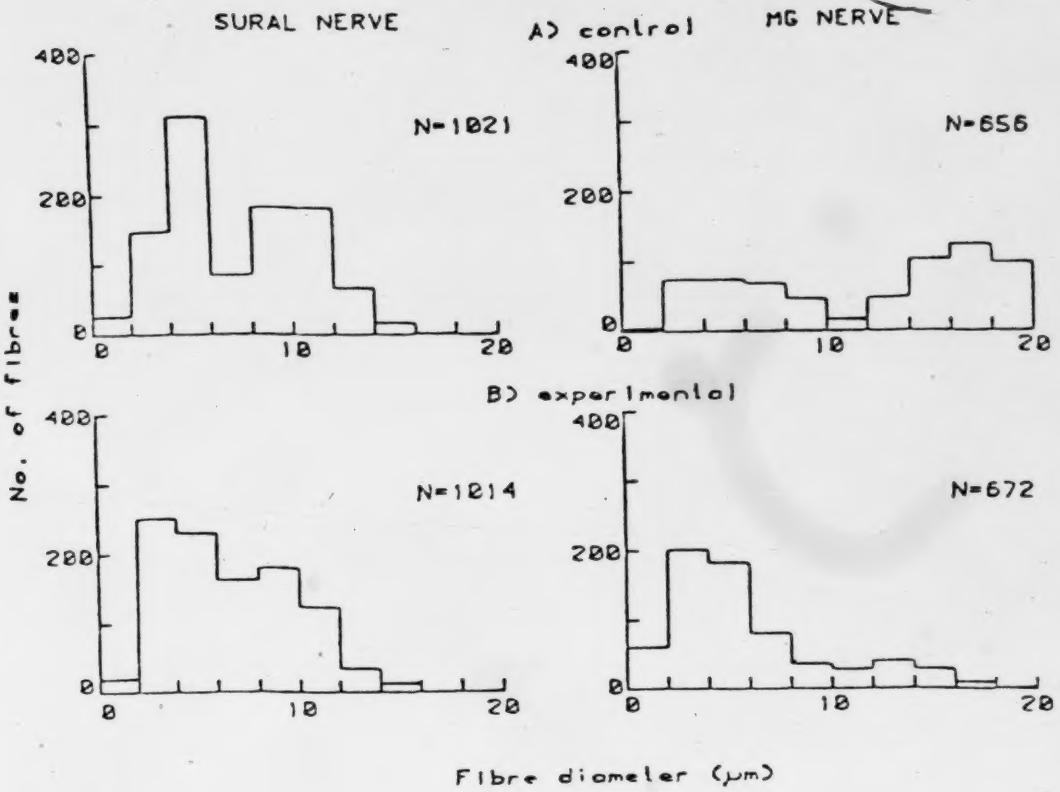


Figure 1.4 Equivalent diameter histograms for the number of fibres in (A) a control sural and medial gastrocnemius (MG) nerves and (B) experimental nerves from an animal studied 203 days after ligation. The total number (N) of fibres in each nerve is indicated.

than 200 days after ligation as described previously. Nerves that had increased numbers of small myelinated fibres indicative of sprouts growing centrally were excluded (Scadding & Thomas 1983). The relative absence of large fibres was not due to a loss of fibres but to atrophy to smaller values. Values for fibres examined 2-3 cm. proximal to the recording site did not differ significantly from the segments examined distally for the nerves included.

Because the bimodal character of the distribution was soon lost during atrophy, the relative changes in small and large fibres were difficult to determine from conventional histograms. There was also considerable variability among the nerves. The results were therefore grouped into four categories as follows:

1. control
2. early experimental (29-71 days after ligation - 3 animals)
3. middle experimental (105-150 days after ligatation - 4 animals)
4. late experimental (198-273 days after ligation - 5 animals).

Average cumulative histograms were plotted for all the nerves (3-5) in any one group and are shown in figure 1.5. For example, if two nerves had 50% and 60% of the fibres less than or equal to 8μm in total diameter, their average value of 55% would be plotted in figure

1.5 for a diameter of 8μm. The use of a logarithmic scale in this figure results in a shift (to the left) of the curve an equal amount when all the fibres atrophy by equal proportions (i.e., reduction from 20μm to 10μm would produce the same shift as a reduction from 10μm to 5μm). The control nerves (x) in each part of figure 1.5 are the contralateral nerves from the same cats from which the experimental nerves were taken. In the early period (∇) little atrophy is evident, although the diameters have shifted a few percent. Atrophy in all fibre groups was seen in the middle and later periods, but still represents a final change of 20% to 30%.

The changes in conduction velocity for these nerves measured by Milner & Stein (1981) showed much larger shifts for all fibre groups when plotted in the same manner (see figure 1.7 below). Since there appeared to be relatively greater atrophy of the axon than of the whole fibre, more detailed measurements were made from electron micrographs, and further comparisons made between the conduction velocity changes and changes in axonal size (cross-sectional area and volume).

Axon Size and Total Fibre Size

From electron micrographs the inner (axon) perimeter and the outer (total fibre: axon + myelin) perimeter were traced and the coordinates automatically entered into a computer (See methods). Variability of repeated measurements was less than 2% and measurements

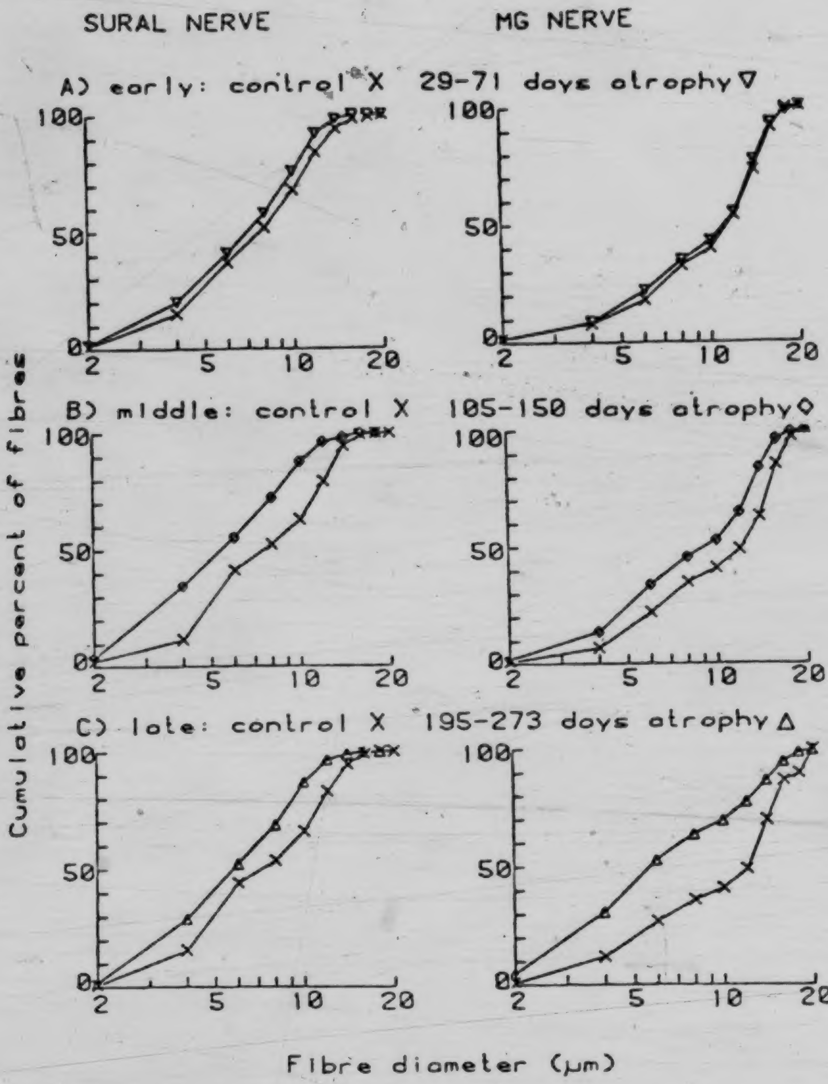


Figure 1.5 Cumulative fibre histograms giving the percentage of fibres less than or equal to the equivalent diameters indicated for control nerves and nerves grouped according to time periods after ligation as indicated. Each cumulative histogram is the average obtained for 2 - 5 nerves. Note that fibre diameters are plotted on a logarithmic scale so that the histograms would shift uniformly to the left, if all fibres atrophied to the same relative extent. Further details in the text.

of perimeter and subsequent calculations of areas and myelin thickness agreed to within 2% or better when compared with direct measurements of myelin thickness. The area within each perimeter was calculated together with the equivalent axon and fibre diameters. Since the experimental nerves are quite irregular and noncircular (see for example the large sural nerve fibres in figure 1.2 and 1.3), the equivalent diameter was taken to be the diameter of a circle having the same cross-sectional area.

The relationships between axon diameter and fibre diameter are plotted in figure 1.6 for control nerves and nerves after middle and later periods of atrophy. Although there is some scatter in the measurements, the data are all fitted by straight lines by least mean squares criteria. The slope of the fitted lines decreases as the time following ligation increases. The slope of the control sural nerve in (A) is 0.66 and in (C) is 0.17. The slopes of the lines for the MG nerve decrease from 0.7 in (A) to 0.41 in (C). Thus, for a fibre diameter of 8 μm, the axon in a control sural nerve would have a diameter of 4-5 μm but only about 2 μm during a later period of atrophy. The changes were somewhat less in the MG nerve, consistent with the less extreme atrophy noted in the light micrographs.

The fitted straight lines also offer a means of converting the fibre diameter histograms of figure 1.4

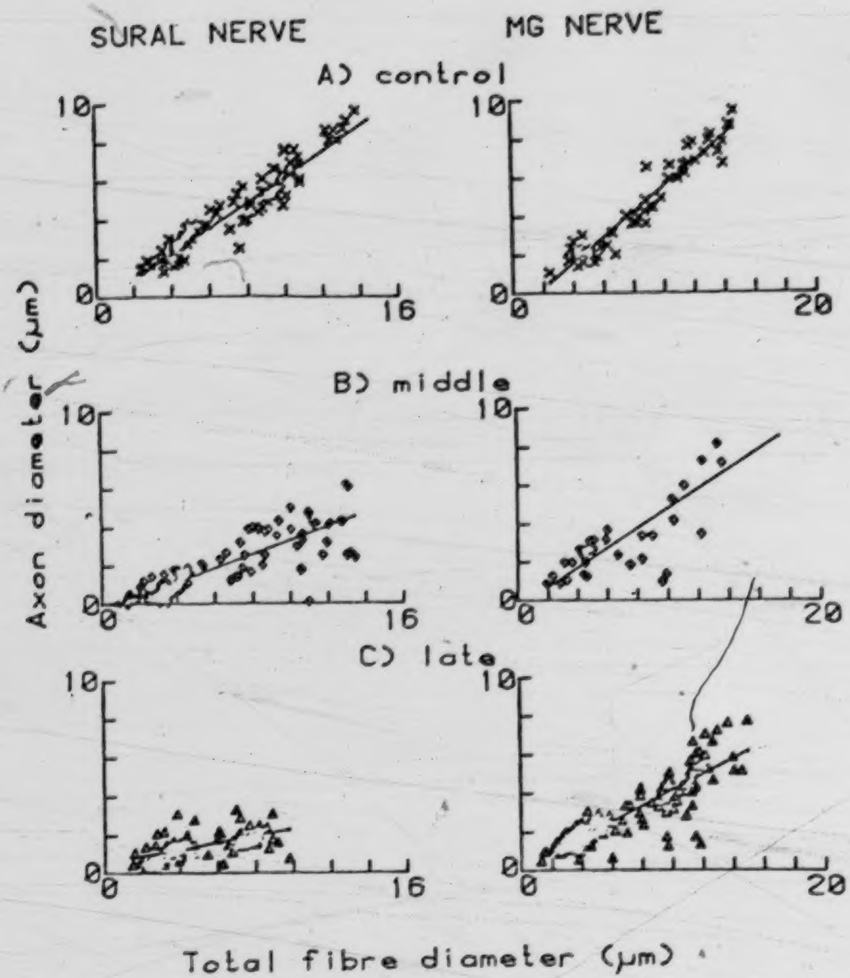


Figure 1.6 Equivalent axon diameter is linearly related to equivalent fibre (axon + myelin) diameter in A) control nerves and at various periods after ligation (B and C), but the slope of the straight line decreases during atrophy. The symbols are the same as in Fig. 1.5. The straight lines were calculated from the standard methods for least mean square deviation and had the following slopes (and standard errors) for the sural nerves A) 0.66 ± 0.02, B) 0.31 ± 0.02, and C) 0.17 ± 0.03, and the MG nerves A) 0.70 ± 0.03, B) 0.51 ± 0.06 C) 0.41 ± 0.02.

to corresponding axon diameter histograms. In figure 1.7 (A) the cumulative fibre diameter histograms are replotted for control (x) nerves and nerves after middle (<>) or later (Δ) periods of atrophy. (Note the change in scale between figures 1.7 (A), (B) and (C). In figure 1.7 (B) cumulative axon diameter histograms are shown after converting from fibre diameter to axon diameter using the slopes of the fitted straight lines in figure 1.6. A much greater relative shift (atrophy) is seen in axon diameter than in fibre diameter.

1.2.2.2 Relation Between Atrophy and Conduction Velocity

The cumulative conduction velocity distributions are plotted in Fig. 1.7 (C) corresponding to the fibre atrophy. (Details of the determination of conduction velocity are provided in Milner & Stein, 1981). In this graph it can be seen, for example, that while only 20% of the fibres in the control sural nerve had conduction velocities equal to or below 20 m/sec, by the late period of atrophy (over 200 days) 50% of the fibres had conduction velocities at or below 20 m/sec. (Conduction velocity has been computed separately for motor and sensory fibres. In order to compare the conduction velocity and fibre diameter histograms, it was assumed that 45% of the fibres in the MG nerve are sensory (Boyd & Davey 1968), and the velocities of sensory and motor combined on this basis). A corresponding 10-fold range of values has been used for the axon diameter, fibre

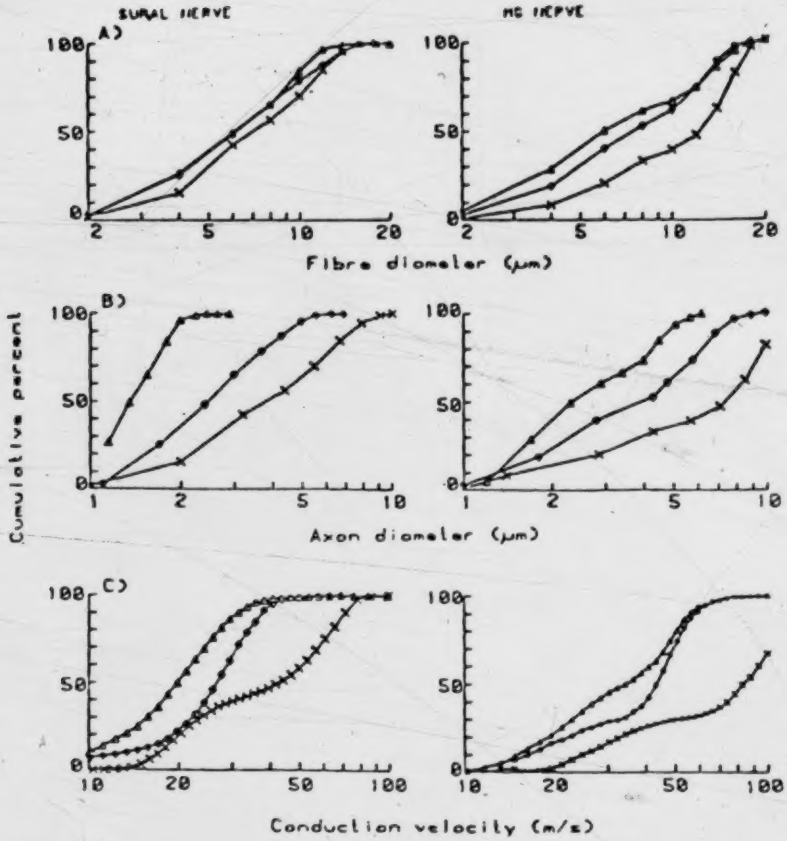


Figure 1.7 Cumulative histograms giving the percentage of sural and MG nerve fibres having A) equivalent fibre diameters, B) equivalent axon diameters and C) conduction velocities less than or equal to the values indicated. The data in A) are the same as in Fig. 1.4 while the data in B) have been transformed using the relationships between axon and fibre diameters shown in Fig. 1.6. Note that different diameter scales used in A) and B), but that a 10-fold range of values is shown on logarithmic scales in all parts of the Figure for ease of comparison. The different symbols are for control nerves (x) and for nerves studied during middle (<>) and late (Δ) periods of atrophy, as in the previous Figures.

diameter and conduction velocity, so that the data can be directly compared as shown in figure 1.7 (A), (B) and (C). It is clear that the change in conduction velocity is considerably greater than the change in the fibre diameter of the nerves, and similar to the change in axon diameter.

1.2.2.3 The Myelin Sheath

Since both increases and decreases in the thickness of the myelin sheath during atrophy have been reported (see the introduction), we wished to test the null hypothesis that no change occurs in the number of turns of myelin (i.e. the thickness of the sheath) or in the length of each turn. The lengths of the innermost and outermost turns are available from the perimeter measurements made on the electron micrographs at magnifications of >5000x (see figure 1.3). The average thickness can be easily computed from the difference between these inner and outer perimeters (see methods and the appendix). From measurements made on the electron micrographs the myelin thickness of the fibres is plotted against the fibre perimeter of the control nerves and nerves after middle (Δ) or late (∇) periods of atrophy in figure 1.8. Arbuthbott et al (1980) reported a logarithmic relationship between myelin thickness and perimeter so a logarithmic scale has been used in plotting perimeter measurements. The relatively linear relationships in the semilogarithmic plots for

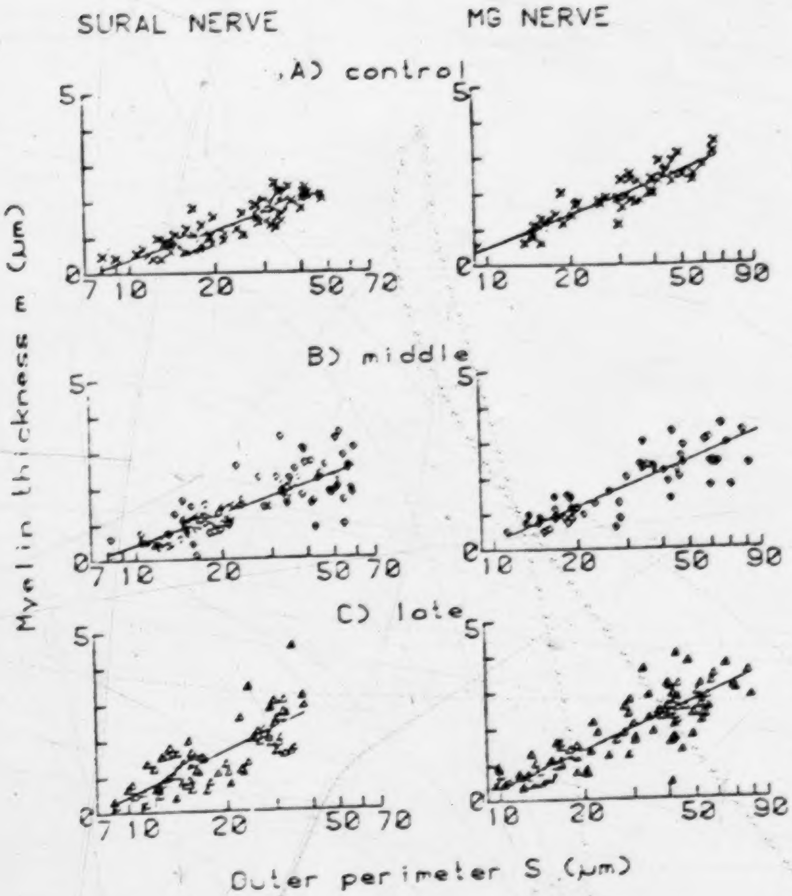


Figure 1.8 Myelin thickness *m* varies approximately as the logarithm of the outer (myelin) perimeter (S) for (A) control nerves and at various stages of atrophy (B) and (C). The slopes of the fitted lines did not show any statistically significant changes over the entire period studied or between the two nerves (c.f. Fig. 1.6). The slope of the control MG line is 3.08 ± 0.19 (ρ=0.92) and the slope of the control sural line is 2.74 ± 0.21 (ρ=0.91) The time periods are as defined in Fig. 1.5.

the control data confirm the earlier results. Furthermore, the relationship appears to hold out to the longest period of atrophy studied. There is no obvious decrease in slope.

1.2.2.4 The ratio *g*

Another parameter of interest is the ratio *g* of inner perimeter to outer perimeter. For a circle the ratio *g* measured from perimeters would be the same as the ratio *d/D* of axon and fibre diameters. Since the axon atrophies substantially for a given fibre diameter (figure 1.6) the ratio *g* measured from perimeters would decline a similar amount if the axons remained relatively circular and the myelin got thicker to fill in the space vacated by the axon. This is clearly not the case as shown in figure 1.9.

Arbuthnott et al (1980) found that the myelin was relatively thinner for the largest fibres (i.e. the ratio *g* was increased). Although there is a wider scatter of values for small compared to large fibres, our data show values of *g* near 0.6 for all sizes of fibres at all stages of atrophy. The value of 0.6 is of interest since it is the value which Rushton (1951) calculated theoretically to optimize conduction of nerve impulses for a circular fibre of a given total diameter. Low values of *g* occurring for the late sural nerves are the result of measurements of axon perimeters in fibres which were so nearly completely closed that their inner

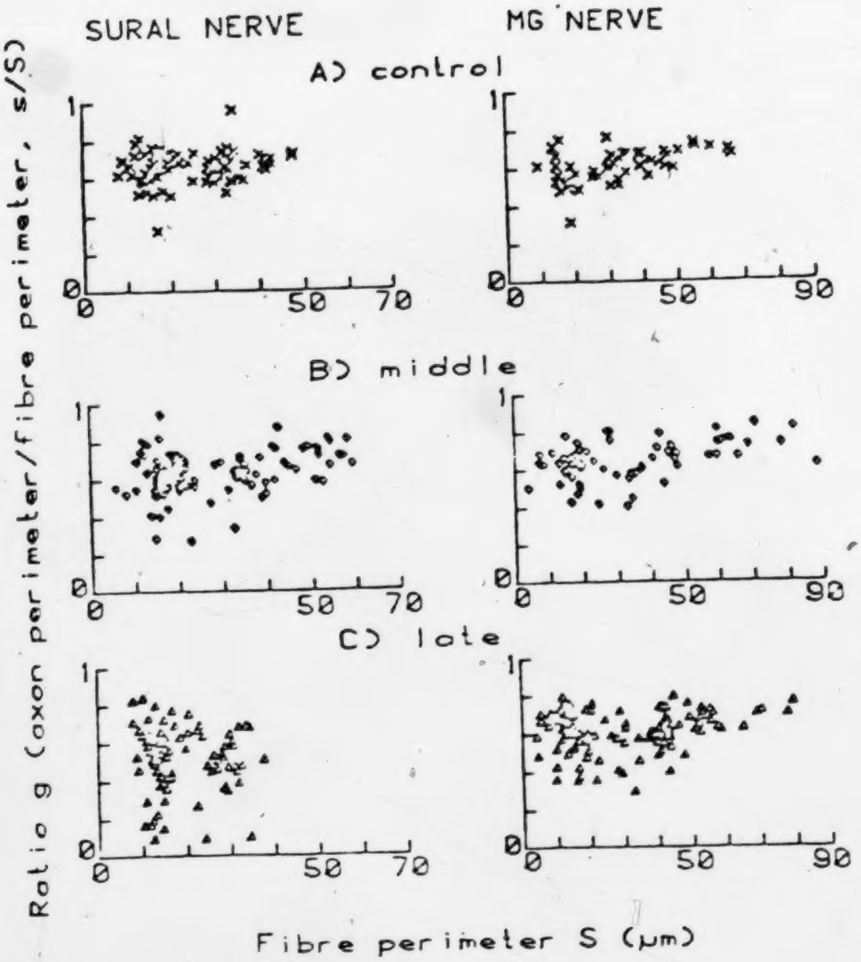


Figure 1.9 Most fibres have a ratio of inner (axon) to outer (myelin) perimeter ($g = s/S$) close to 0.6 in (A) control nerves and nerves at various periods of atrophy (B) and (C). The scatter of values tends to be greater for small fibres and after long periods of atrophy owing to masking of the perimeter when fibres are nearly completely closed. The time periods are as defined in Fig.1.5.

(axon) perimeters were pressed together and therefore more difficult to trace accurately.

Another possibility is segmental demyelination but in this case the values of *g* should approach 1, which was not observed. Thus, these measurements of myelin thickness and the ratio *g* are consistent with the null hypothesis that there are no substantial morphometric changes in the myelin except perhaps for those few fibres showing the most extreme degree of atrophy.

1.2.2.5 Non-circularity

Arbuthnott et al (1980) found that nerve axons are normally non-circular and provided a normalized index of circularity, which can be applied to axons or fibres (axon + myelin), as indicated in the Methods. Our average values for the circularity of control fibres were all between 0.7 and 0.8 as shown in figure 1.10 while the circularity of control axons was about 0.2 lower for large and small fibres. The circularity decreases steadily with time after nerve ligation so that the average values are near 0.3 for axons after the longest periods of atrophy. These values for control fibres are lower than the values of 0.80 for large fibres and 0.77 for small fibres reported by Arbuthnott et al (1980), who had excluded axons sectioned in para-nodal regions or at Schmitt-Lantermann clefts. Correction of our measurements for control nerves brings the circularity of large fibres in MG nerve from 0.78 to

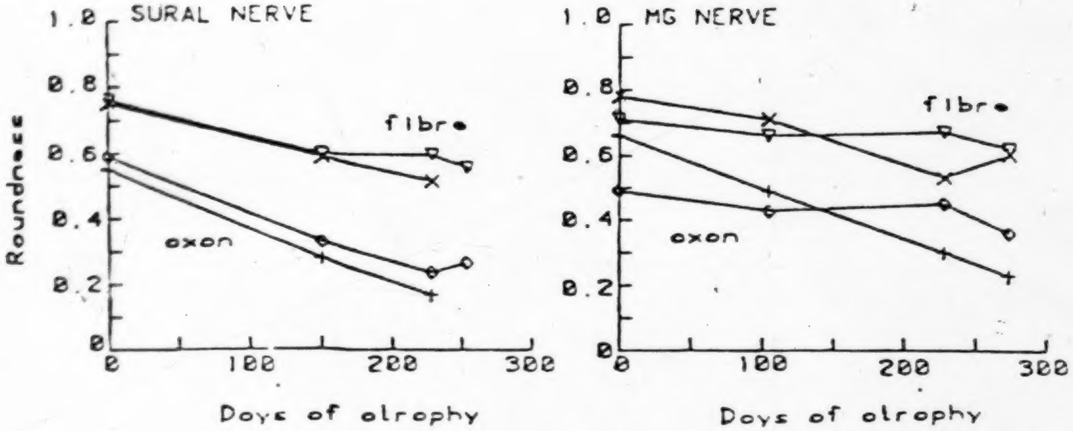


Figure. 1.10 The circularity of axons and fibres (axon + myelin) in sural and MG nerves decreases steadily with time after nerves are ligated. No major differences in the circularity of large (x,+) and small (Δ,<>) fibres were seen at any time period, but the circularity of the axons was always less than that of the fibres. (Error bars fall within the symbols).

0.92 and that of small fibres from 0.71 to 0.84. Since so many of the atrophied fibres had the same appearance as normal fibres at nodes and Schmitt-Lantermann clefts this correction cannot be made for the experimental nerves. For comparison see figure 1.12 (C) (and the appendix) which shows an axon with the long axis 2.5 times longer than the short axis. This represents an index of circularity of 0.76.

Thus these measurements of myelin thickness and the ratio *g* confirm that although the axons become increasingly non-circular, and the axon shrinks, there are minimal morphometric changes in the myelin sheath during the atrophy of peripheral nerves.

1.2.2.6 Computed Conduction Velocity

Changes in conduction velocity were calculated based on the Frankenhaeuser-Huxley equations for frog Nodes of Ranvier (see Methods). Although there are important differences between frog nodes and mammalian nodes (Ritchie 1979; Chiu & Ritchie 1980), analogous equations are not available for mammalian nodes. Changes in axoplasmic resistance such as would occur during atrophy were entered and the action potentials computed for the fifth (of ten) nodes as shown in figure 1.11 (A). A wide variation in axoplasmic resistance or equivalent axon diameter had relatively little effect on the size or duration of the spike, but produced a marked increase in the time for the action potential

Figure 1.11 (A) Action potentials computed from the Frankenhaeuser-Huxley equations for a frog myelinated fibre. As axoplasmic resistance per unit length of cable is increased (resulting from atrophy of the axon), the time to conduct to the fifth node (whose action potentials are shown here) slows markedly. From left to right the values of axoplasmic resistance are 1, 4, 6 and 8 times the standard values (see Methods), corresponding to a decrease in equivalent axon diameter to 1, 0.5, 0.41 and 0.35 times the standard value of 10.5 µm For smaller values of diameter (higher axoplasmic resistances) conduction failed altogether. Note the slow depolarization which just reaches threshold for the action potential on the far right of (A). (B) The conduction velocity of the action potentials shown in (A) varies approximately linearly with the equivalent axon diameter (x) or fibre (axon + myelin) diameter (¤). However, a three-fold change in conduction velocity (from 15 to 5 m/s) is associated with about a two-fold change in axon diameter, but less than an 20% change in fibre diameter (from 13 to 11 µm). The y-intercept of the line for the relation of conduction velocity to axon diameter d is 2.3 µm while the y-intercept for the outer fibre diameter D is 10.4 µm.

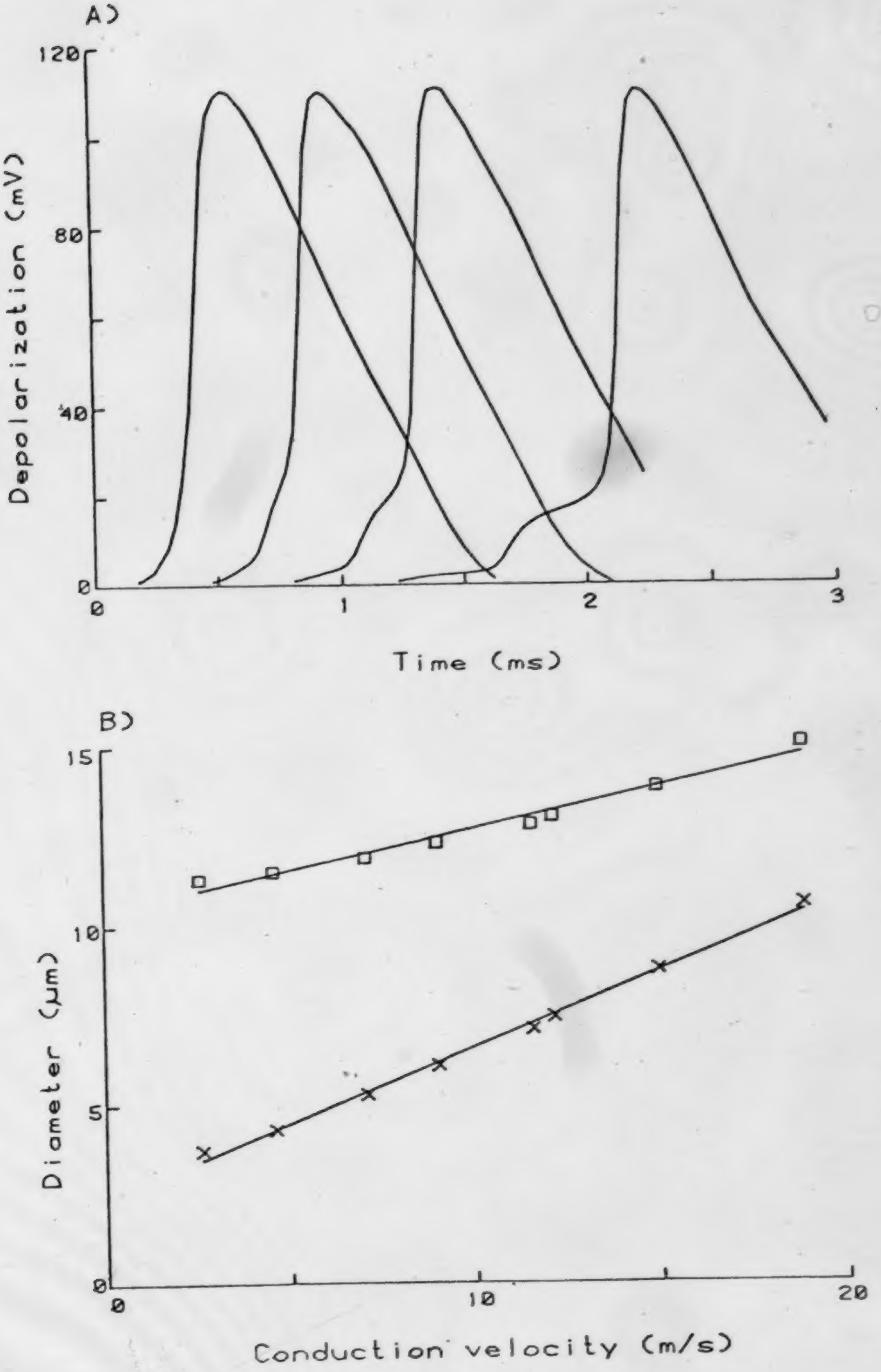

A)
120
80
40
0
Depolarization (mV)
0
1
2
3
Time (ms)
B)
15
10
5
0
Diameter (μm)
0
10
20
Conduction velocity (m/s)

Fig. 1.11

to propagate to this point in the fibre. As the axoplasmic resistance increases it becomes more difficult for the action potential to spread along the membrane and with the highest value shown, the voltage just reaches threshold. Conduction failed when the axoplasmic resistance was increased to more than 8 times the standard value. Although nodal membrane properties were assumed to remain unchanged, conduction velocity is relatively insensitive to nodal properties as compared to internodal properties (Boyd 1964; 1966). If there were segmental demyelination the above calculations would not apply (Koles & Rasminsky 1972).

Conduction velocity was computed from the propagation time between nodes (see Methods) and the observed values are plotted for different values of the equivalent axon diameter *d* eq and fibre diameter *D* eq (Fig. 1.11 B). If conduction velocity changed proportionally with equivalent diameter a straight line through the origin would result. Although neither fitted straight line goes precisely through the origin, the approximation is much better for the axon diameter *d* than the fibre diameter *D* (the intercept on the y-axis is 10.4 μm for *D* and only 2.3 μm for *d*). Thus, functional changes should be more nearly correlated with changes in axon diameter than total fibre diameter, as found experimentally.

1.2.3 Discussion

These results indicate clearly that measurements of outer fibre diameter underestimate the changes in fibre morphology and the ability of axons to conduct action potentials which take place during atrophy. As the axons became more shrunken with time after ligating a nerve, the intact tube formed by the Schwann cell and myelin appeared merely to collapse inward (Causey 1948; Spencer & Lieberman 1971; Dyck et al 1980; 1981). Thus, the changes that took place in fibre diameter were much less than those occurring in axon diameter (Fig.1.6). The conduction velocity of the nerve fibres was related more closely to axon diameter than total fibre diameter during the course of atrophy. In order therefore to determine the functional effects of nerve atrophy from anatomical measurements, electron microscopy must be used to measure axon size accurately.

Early work using light micrographs was inconclusive as to whether the myelin thickness increased or decreased during atrophy of the axon (see Introduction). From the present measurements from electron micrographs, there is no evidence that the myelin changes in average thickness (Fig.1.8) despite the substantial surface changes (Spencer & Lieberman 1971; Dyck et al 1981). Furthermore, the relationship between inner (axon) perimeter and outer (myelin) perimeter remained unchanged. The data suggest that these parameters and relationships are quite insensitive to the gross morphological changes taking place in the axonal geometry.

How can the myelin thickness (or the number of turns) and the perimeter (or the length of individual turns) remain invariant without buckling and separating from the axon during the course of atrophy? In the Appendix a simple scheme is presented in which it is possible for the axon to disappear completely without the inner perimeter, the outer perimeter or the myelin thickness changing. The circular shape in Figure 1.12 (A) evolves to the slit in Figure 1.12 (B) via a series of increasingly flat shapes of the type shown in Figure 1.12 (C).

The result is generalized in Figure 1.12 (D) to any arbitrarily shaped polygon as long as extreme infolding does not occur (Figure 1.12 E)). Thus, there is a broad class of paths an axon could follow during atrophy without requiring the myelin to alter its thickness or perimeter. Only in the terminal stages of atrophy, when the axons show severe enough infolding to produce sharp angles, must reorganization of the myelin occur in a way which will produce a separation in the myelin layers. Our experimental results strongly support this process for atrophy.

A further consequence of this argument is that the axon could regrow to its original size by simply reversing the geometric changes, if regeneration is permitted. Gordon and Stein (1982) have recently shown that the size and conduction velocity of axons proximal to a lesion does return precisely to control levels if and only if

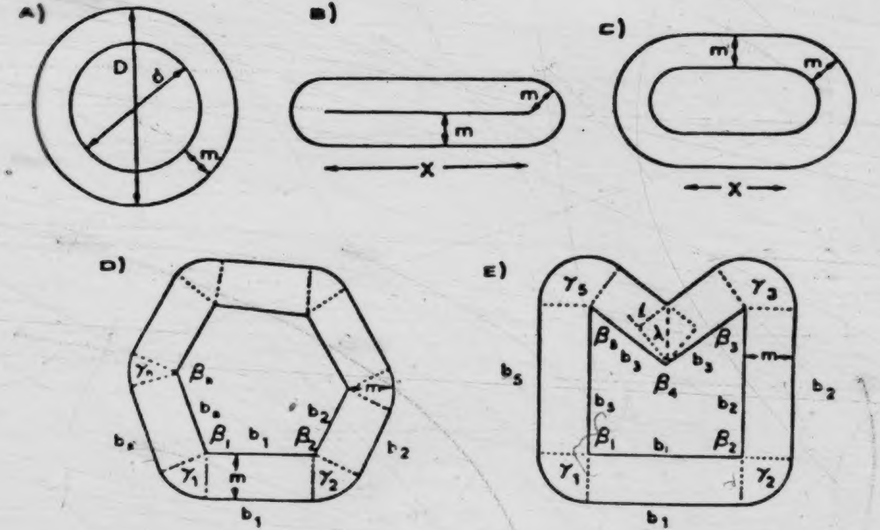

A)
D
d
m
B)
m
m
X
C)
m
m
X
D)
γ_n
β_n
b_n
b_2
β_1
b_1
β_2
m
b_n
b_2
γ_1
m
γ_2
b_1
E)
γ_5
l
λ
γ_3
β_5
b_5
b_3
β_3
m
β_4
b_5
b_2
b_3
b_2
β_1
b_1
β_2
γ_1
γ_2
b_1

Fig. 1.12 A myelinated nerve fibre could pass from a circular shape (A) to a slit (B; no axon) via a series of flattened shapes (C) without changing the myelin thickness *m*, the inner perimeter or the outer perimeter. *D* = outer fibre diameter, *d* = inner axon diameter, *x* = length of flattened portion and *y* = short axis of the axon. Any arbitrarily shaped axon (D) can be approximated by a polygon to a desired degree of accuracy. Myelin of constant thickness *m* can then be laid down such that the outer perimeter S is related to the inner perimeter *s* by the relation $S = s + 2 m$, as long as the angles of the polygon are all less than π radians. If there are obtuse angles greater than π radians (E), then myelin of constant thickness can not be laid down and the relation between the inner and outer perimeters (*s* and *S*) cited above no longer holds exactly. See the Appendix for definition of symbols and derivation of these relationships.

regeneration to a suitable end organ occurs (see also Cragg & Thomas 1961). In contrast, if the myelin were to expand and replace the space vacated by the axon, it could be much more difficult for the axon to regain its former size.

Finally, because the axon is not normally circular, it could hypertrophy so that circularity increases from 0.7 or 0.8 to 1.0, without causing any severe complications in the myelin. This also provides a margin of safety for the axon against osmotic shock or other local changes and provides a rationale for the normal noncircularity of axons. Our experimental and theoretical results are all consistent with a simple, reversible mode of nerve axon atrophy and hypertrophy. There appears to be no stimulus for the myelin to disintegrate nor for the the Schwann cells of the sheath to separate or divide or to phagocytize the myelin. As an axon loses volume during atrophy, tissue fluid pressure outside the myelin flattens it in such a way that there is no change in the number of turns of myelin or the length of each turn. The axonal conduction velocity decreases approximately in parallel with the decrease in effective axonal diameter (but not the external fibre diameter). If regeneration occurs, the process reverses along a similar geometric path to its original size or possibly to a more circular, hypertrophied size.

2. THE REINNERVATION OF MUSCLE

2.1 MUSCLE AND MOTOR UNIT PROPERTIES AFTER NON-SPECIFIC REINNERVATION OF FAST AND SLOW MUSCLE

2.1.1 Introduction

2.1.1.1 Re-growth of the Nerve

Within a short time after nerve injury and the development of the axon reaction (see page 4), sprouts will begin to grow from the cut end of the proximal segment and from the adjacent nodes. These are seen to develop a "growth cone" or terminal swelling which precedes the sprout towards the site of injury and scar formation. (Ramon y Cajal 1928; Yamada 1971; Morris, Hudson & Weddell 1972b). Morris et al (1972b) described the appearance of a "regenerating unit" comprised of an original myelinated axon and a number of its collateral sprouts closely associated with each other and with one or a few Schwann cells, all surrounded by a single basal lamina. The growing axons and their branches either enter a single endoneurial tube or its collateral sprouts and are seen to adhere to and grow along the external surface of a tube, all surrounded by a basal lamina. While sprouts are seen to diverge, turn aside or back (Scadding & Thomas 1983) and coil to form neuromas, the majority penetrate the scar tissue and after a delay of 1-2 days crossing the scar, grow towards their original target tissue at a constant rate which is reported to be in the rat 2-3 mm per day (Grafstein & McQuarrie 1978). Regenerating axons are seen to branch during their progress towards the periphery (Cajal 1928; Eccles & Sherrington 1930). Regenerating axons that make successful contact with the periphery mature and become remyelinated, enlarging back towards their original size (Sanders & Whitteridge 1946; Cragg & Thomas 1961). It is not known what proportion of the multiple sprouts formed survive.

2.1.1.2 Nerve-Muscle Specificity

Among the factors playing an important role in the achievement of precise, delicate control of movement are the specific location of the motor neuron in the spinal cord (Romanes 1964), the highly specific connections formed during embryogenesis between motor neurons and muscles (Landmesser & Morris 1975) and the ordered recruitment of motor neurons during the development of muscle contraction (Henneman & Olson 1965). It has been shown that during development the outgrowing axon shows a high degree of selectivity in its choice of target. Axons of motor neurons growing towards a limb maintain their anterior-posterior topographical order (Landmesser 1980) and seldom enter an inappropriate muscle (Landmesser & Morris 1975). The position of the cell body in the spinal cord correlates with the site of termination of the axon, and it appears that neurons are specified with respect to their peripheral target prior to outgrowth of the axon (Sperry 1943, cited in Gaze 1974; Landmesser & Morris 1975).

The specificity conferred on developing cells may be expressed by chemical markers residing in the "labelled surfaces" of astrocytes or Schwann cells or their basal laminae (Fallon & Mcraff 1982; Smallheiser, Crain & Reid 1982), by mechanical guidance by support cell membranes (Letourneau 1975); or the attraction towards a specific target may come from a trophic substance released by the target cell. It also appears that, while mechanical guidance of the growing axons may play a role, the axon can actively respond to environmental cues in seeking its correct termination. 'Pioneer pathfinder cells' have recently been shown in the grasshopper (Ho et al 1982; Snipes, Mayes & Freeman 1982; Smalheiser, Crain & Reid 1982) which may play a role in the specificity shown by the developing axon by leading it's growth towards its correct target.

A trophic substance necessary to support the outgrowth of axons of autonomic and dorsal root ganglion cells, and the survival of autonomic cells in the adult has been clearly demonstrated (Levi-Montalcini & Angeletti 1968; Varon 1975). This nerve growth factor is pinocytosed and retrogradely transported to the cell (Hendry et al 1974; Stackel & Theonen 1975; Schwab & Theonen 1976). No corresponding "nerve growth factor" has been unequivocally demonstrated for the somatic cells of the spinal cord but there is increasing evidence to suggest that a similar trophic substance is released from muscle. A non-dialyzable factor from heart muscle cells supports the survival and growth of cultured chick ciliary ganglion cells (Landmesser & Pilar 1976), and similarly a non-dialyzable substance is provided by epithelial cells of mouse submandibular gland which supports growth and branching of axons of cholinergic nerves (Coughlin 1975). Cultures of fetal mouse spinal cord show increased choline acetyl-transferase (CAT) when co-cultured with skeletal muscle cells or in a medium conditioned by skeletal muscle cells (Giller et al 1977; reviewed by Varon & Bunge 1978). Riopelle & Cameron (1982) have recently demonstrated the presence of a trophic substance for alpha motor neurons in culture medium conditioned by autologous tissues.

Guidance of the regenerating axon towards its original target is thought to be supplied by at least two of the same factors - contact guidance and chemoaffinity. After nerve crush, the regenerating axons grow down the endoneurial connective tissue tubes of the distal segment. In this case they grow down their own endoneurium to the correct target muscle. In the case of nerve section the periphery i.e. the muscle and/or the Schwann cells which are present in the endoneurial tubes

(Longo & Penhoet 1974; Smalheiser,Crain & Reid 1982) may supply a trophic substance to attract the sprouts (Varon 1975). However, the high degree of embryonic specificity of neuron for muscle fibre can no longer be seen to act during regeneration, and axons can readily innervate a foreign muscle without necessarily regrowing to the appropriate region of the limb (Gutmann & Young 1944; Weiss & Hoag 1946).

2.1.1.3 Properties of Normal Muscle

Contractile Properties of Muscle. Adult mammalian twitch muscles have differing contractile characteristics, and generally can be categorized as either fast, phasically acting muscles or slow, tonically contracting muscles (Eccles, Eccles & Lundberg 1958; Lewis & Ridge 1981). At birth all muscle fibres in the cat and rat are slow (Buller, Eccles & Eccles 1960; Brooke, Williamson & Kaiser 1971; Hammarberg & Kellerth 1975; Kugelberg 1976). During the first five to six weeks of life the muscles differentiate, contraction times shorten and fast muscle fibres reach their adult values of contraction speed by six weeks. There is then a progressive slowing of slow muscles over the following 16 to 20 weeks before they attain the contraction speed of adult muscle (Buller et al 1960; Close 1964; Karpati & Engel 1967; Brooke, Williamson & Kaiser 1970; Kugelberg 1976).

Spinal motor neurons undergo postnatal differentiation into slow and fast types over the early weeks of life (Sato, Mizumo & Konishi 1977), during the time when there is also elimination of polyneuronal innervation of muscle fibres (Bennett & Pettigrew 1974; Brown, Jansen & Van Essen 1976) leaving adult muscle fibres slow or fast in accordance with the type of neuron innvervating the fibre.

Fibre Composition of Muscle The enzyme profile of individual muscle fibres can be demonstrated by histochemical staining of muscle cross-sections to identify oxidative and glycolytic enzymes, and the myosin ATPase activity. The myosin ATPases of fast and slow muscle can be differentially demonstrated by pre-incubation at acid and alkaline pH (Guth & Samaha 1969, 1970). Adult muscles may be relatively homogeneous, containing predominantly one type of fibre, or they may be heterogeneous, made up of a mosaic of fibre types (Ariano et al 1973). Fast contracting (pale) muscles are generally heterogenous with respect to fibre composition while slow muscles are red and have a more homogenous fibre population. A typically fast muscle such as the gastrocnemius contains a mosaic of fast, slow and intermediate fibres in both the cat and rat (Ariano et al 1973) while the slow postural muscles such as soleus tend to have for example, 80% slow fibres in the rat (Close 1969) and 100% slow fibres in the cat

(Ariano et al 1973).

2.1.1.4 The Motor Unit

The concept that the motor unit - "the motor neuron and all the muscle fibres innervated by it" is the functional unit of muscle was first proposed by Sherrington (1929). From that time, investigations of the properties of muscle (reviewed by Close 1972) expanded to include investigation of the motor unit population and the properties of the motor units of individual muscles (Eccles & Sherrington 1930; Burke et al 1977; Chan et al 1982; Dum et al 1982). Eccles et al (1958) noted differences between the alpha motor neurons supplying fast and slow muscles, and subsequent research into the contractile properties of motor units in the cat (Eccles et al 1960; Olson & Swett 1960; Wuerker, McPhedran & Henneman 1965; Hammarberg & Kellerth 1975), the rat (Close 1967; Edstrom & Kugelberg 1968) and man (McComas & Sica 1973) established that there are functional types of motor units (either fast or slow) and the properties indicated that muscle fibres belonging to the motor unit were probably homogeneous in type (Wuerker, McPhedran & Henneman 1965; Kugelberg 1968). The introduction of histochemical techniques to identify muscle fibre types (Dubowitz & Pearse 1960; Edstrom & Kugelberg 1968; Brooke & Kaiser 1970) according to their oxidative enzyme profile and their myosin-ATPase activity (Stein & Padykula 1962; Guth &

Samaha 1969) was combined with the the application of the glycogen depletion technique (stimulation of a single motor nerve fibre until the muscle motor unit fibres were depleted of stored glycogen and identification of the muscle fibres of the motor unit by the periodic-acid Schiff (PAS) reaction) to show that "the histochemical composition of fibres in a single motor unit was uniform" (Edstrom & Kugelberg 1968). The PAS technique was also used to demonstrate that fibres of a single motor unit are scattered over 10-30% of the cross-sectional area of the muscle (Edstrom & Kugelberg 1968) intermingled in a mosaic with fibres of other motor units. Motor unit territories covering 25% of the area of a muscle were determined by electrophysiological recording (Buchthal & Rosenfalck 1973; Brandstater & Lambert 1973) and Burke & Tsairis (1973) reported territories of 40 to 50% of the muscle determined by electrophysiology and histology.

There is normally a high correlation between the histochemical profile and the contractile characteristics of individual motor units. Descriptions of motor unit contractile characteristics tend to imply that there will be a related group of histochemical characteristics (Dubowitz & Pearse 1960; Edstrom & Kugelberg 1968; Brooke & Kaiser 1970; Peter, Barnard, Edgerton, Gillespie & Stempel 1972; Burke, Levine, Tsairis & Zajac 1973; Warmoltz & Engel 1973). One unfortunate outcome of the burgeoning work in this field was some confusion resulting from multiple terminologies (Brooke & Kaiser 1969; discussed by Peter et al 1972), the most commonly used being: (a) Type I (slow) and Type II (fast) (Dubowitz & Pearse 1960), (b) Type I (slow), Type IIa and IIb (fast-oxidative -glycolytic and fast-glycolytic)(Brooke & Kaiser 1970), (c) intermediate, red and white (Padykula & Gauthier 1966), (d) type B, C and A (Stein & Padykula 1962), and (e) slow, f.o.g. (fast-oxidative-glycolytic) and f.g. (fast-glycolytic), (Peter et al 1972) which correspond to type S (slow) FR (Fast, Fatigue-Resistant) and FF (Fast-Fatiguable) physiological motor unit types (Burke et al 1973).

2.1.1.5 Denervated Muscle

There are several major consequences of denervation of a muscle (reviewed by Thesleff 1974; Harris 1980). During the early weeks the muscle fibrillates and later there is gross atrophy (Fischer 1939; duBois & Almon 1981). Changes in the membrane lead to an increase in specific membrane resistance, (Nicholls 1956), a decrease in the resting membrane potential (Stanley & Drachman 1980) and an increase in extra-junctional sensitivity to acetylcholine (Kuffler 1943). In time the unoccupied neuromuscular junctions degenerate (Miledi & Slater 1970). The increased ACh sensitivity is due to the appearance of new receptors outside the original end-plate region (Axelsson & Thesleff 1959; Miledi 1960), It was suggested that this was a result of the release of some trophic substance from the degenerating nerve (Oh et al 1980; Guth et al 1980) rather than the loss of a trophic influence from the nerve. (A trophic substance present in nerve which supports muscle cells has recently been demonstrated. A soluble protein present in both normal and degenerating peripheral (sciatic) nerves enhanced survival and maturation of myotubes in culture (Oh et al 1980a and b) and a sciatic nerve extract reduced the weight loss in denervated rat muscle (Davis & Kiernan 1980)).

The contractile characteristics of muscle are also altered by denervation. Muscles showed increased time to peak tension, reduced tetanic tension and absolute twitch tension. The tetanic tension decreased relatively more than the twitch tension, therefore muscles had an increased twitch/tetanic ratio (Kean et al 1974). Progressive slowing of the time to peak and of the half-relaxation time of the twitch and the relaxation of the tetanus of fast muscle was produced by either denervation or inactivity after the application of botulinum toxin in rat EDL. (Drachman and Johnston 1975). Similar changes in cat FDL resulted from prolonged inactivity (sleep) or spinal cord isolation (Davis & Montgomery 1975). Both studies reported similar but less pronounced changes in the slow soleus.

2.1.1.6 Reinnervation of Muscle

Synaptogenesis. Normally innervated adult muscle lacks extra-junctional ACh sensitivity and will not accept foreign or supernumerary innervation (Jansen et al 1973). Reinnervation of a denervated muscle reduces the extrajunctional Ach sensitivity (Miledi 1960; Bennett, Pettigrew & Taylor 1973) and restores the refractoriness of the muscle to further synaptogenesis. However, neither reinnervation at a newly-formed end-plate by a foreign nerve (Gutmann & Hanslikova 1967) nor electrical stimulation of denervated muscle (Jansen et al 1973) prevents reinnervation of muscle by its own nerve. Synaptogenesis during reinnervation of a muscle either by its own or a foreign nerve or as a result of intramuscular collateral sprouting (Edds 1953) commonly occurs at the original end-plate site (Miledi 1960; Frank et al 1975; Bennett & Raftos 1977; Brown & Ironton 1978) where at least some of the cues to the axon reside in the muscle basal lamina (Sanes et al 1978). Multi-innervation during reinnervation may occur transiently as a result of either several axon sprouts occupying the same endplate or the formation of new end-plates by the foreign nerve (Frank et al 1975; Bennett & Raftos 1977). The return of the original nerve to the myofibres normally causes suppression or elimination of the foreign synapses within a few weeks. (Benoit & Changeux 1978; Brown & Ironton 1978). However

Frank et al (1975) were able to demonstrate persisting foreign innervation of rat soleus muscle both on widely-separated end-plates and as dually innervated original end-plates after reinnervation of the muscle by its own nerve. The returning original nerve displayed no ability to suppress the foreign synapses in that instance.

2.1.1.7 The Effect of Reinnervation or Activity on Muscle Properties

It was noted by Fischer (1939) that the atrophy and changes in excitability caused by denervation of muscle could be retarded by stimulation of muscle. Later experiments (Lømo & Rosenthal 1972) revealed that atrophy similar to that caused by denervation could be produced by muscle inactivity produced by blockade of the nerve with anaesthetic or diphtheria toxin, and that such blockade caused the entire muscle membrane to become sensitive to iontophoretically applied acetylcholine. Lømo & Westgaard (1975) used direct stimulation of muscle to reverse the increased extrajunctional sensitivity of denervated muscle membrane. The effectiveness of the stimulation was related to the pattern and amount of stimulation. Higher frequencies and amounts of stimulation were more effective than slower less frequent stimuli. The increase in sensitivity could be prevented by chronic stimulation of the nerve distal to the block, when neuromuscular transmission remained unimpaired.

Drachman and Johnston (1975) showed that denervation and inactivity caused similar progressive slowing of muscle. It was subsequently shown by Herbison, Jaweed & Ditunno (1981) that denervation-evoked changes in muscle contractile properties were reversed by reinnervation and by muscle activity.

Vrbova (1963) found that the impulse activity of motor neurons affected the speed of contraction of striated muscle. Chronic low frequency long-term stimulation has been shown to cause a fast muscle to become indistinguishable from slow muscle by a number of physiological, biochemical and histochemical criteria (Lømo & Westgaard 1975; Salmons & Sreter 1976; Jaweed, Herbison & Ditunno 1982). Long-term phasic stimulation at slow frequencies (2.5 Hz) will also effect fast to slow transformation of fast muscle (Sreter et al 1982) suggesting that muscle fibre transformation depends not only on the pattern of stimulation but also on the total amount of activity. The effects of chronic fast stimulation on slow muscle fibre is less well established (Lømo et al 1980) but clearly the pattern of activity influences the changes observed.

2.1.1.8 Cross-Innervation Between Fast and Slow Muscles

Although denervated muscle will accept reinnervation by its own and other motor fibres, it has been shown that sensory nerve fibres cannot establish functional contact with muscle fibres (Gutmann 1945; Weiss & Edds 1945). Reinnervation of muscle by a foreign nerve does not alter the properties of the motor neurons, (Sperry 1941; Buller, Eccles & Eccles 1960; Eccles et al 1962; Kuno et al 1974) nor is there reorganization of the afferent connections of the motor neuron pool (Sperry 1941; Buller, Eccles & Eccles 1960; Mendell & Scott 1975).

Cross-union of the nerves of fast and slow muscles changes some of the properties of the muscles. The contractile speeds are altered in such a way that when a fast muscle nerve innervates a slow muscle the muscle becomes faster and a slow muscle nerve innervating fast muscle causes the muscle to become slower (Buller, Eccles & Eccles 1960; Close 1965, 1969; Dubowitz 1967). The slow muscle is more resistant to change than the fast (Buller et al 1960; Dubowitz 1967).

The enzyme profile of cross-innervated muscle is altered (Romanul & Van Der Meulen 1966; Yellin 1967; Essen, Jansson et al 1975) followed by alterations in the myosin molecule (Barany & Close 1971; Reichmann et al 1983). Myosin ATPase was found to contain both fast-and slow-type light chains within a single fibre (Sreter et al 1980; Gauthier et al 1983).
Cross-innervated muscle also shows a reorganization of motor unit fibre distribution (Kugelberg 1973).

Histochemical enzyme staining may show "type-gouping" in which fibres of the same histochemical type and of the same motor unit are found in a large "clump" (Yellin 1967; Karpati & Engel 1968a; Kugelberg, Edstrom & Abbruzzese 1970). Some ulstrastructural parameters which can be used to discriminate between fast and slow muscle (Eisenberg & Kuda 1976; Beringer 1976; Ellisman et al 1976) were altered in muscle in accordance with altered innervation (Ellisman et al 1978).

2.1.1.9 Competition Between Nerve and Muscle Fibres During Reinnervation

The plasticity of muscle fibres after reinnervation demonstrates that muscle fibres accept innervation by nerves of a different type than their own original nerve. Early experiments in which cross-innervation of fast and slow muscle and/or electrical stimulation caused changes in muscle properties led to the conclusion that the trophic influence of the nerve controlled many of the properties of the muscle fibre (Buller, Eccles & Eccles 1960). Later experiments attempted to determine whether muscle fibres would be preferentially reinnervated by their own original type of nerve fibre (Weiss & Hoag 1946; Bernstein & Guth 1961; Miledi & Stefani 1969; Hoh 1975). Conclusions of these experiments were that nerve-muscle specificity did not act to control the process of reinnervation of muscle. The next two chapters will describe experiments which examined in more detail the exact innervation of individual muscles and the properties of the muscles and of their motor unit population after reinnervation by a nerve which allowed competition between the muscles' own original motor axons and axons of the opposite type.

2.2 RANDOM REINNERVATION OF SLOW AND FAST MUSCLE

2.2.1 Introduction

Recovery of efficient motor control following nerve injury would be greatly enhanced if it were possible to redirect growing axons to their original target. In view of the mode of growth of regenerating axons down pre-existing endoneurial tubes, the establishment of correct connections would be dependent on axons entering their former pathways. If axons indiscriminately enter any available endoneurial tube encountered by the growth cone, then in order to achieve correct reinnervation muscles cells must retain some kind of identity that is recognizable by the nerve, or the muscle must reject inappropriate synapses. The problem of correct reinnervation is particularly severe when injury has damaged a large nerve trunk in the limbs, denervating many muscles.

During embryogenesis motor neurons and muscles develop highly specific connections based on the position of the neuron in the neuraxis, environmental cues and contact guidance of the developing axon, and possibly a biochemical identity for both cells (reviewed by Landmesser 1980). Early experiments attempted to demonstrate whether nerve-muscle specificity could still act in the adult to ensure correct reinnervation of muscles by their former nerves after injury. Offering all the axons of a nerve trunk to all the denervated muscles of the limb distal to a nerve cut and repair (Sperry 1941; Weiss & Hoag 1946; Bernstein & Guth 1961) resembled the situation caused by injury to a large nerve trunk. Results from this early work were not conclusive and did not provide information on the characteristics of the muscles before and after reinnervation. It was difficult to glean much information regarding which axons innervated which muscles or to determine whether fast and slow muscle fibres were preferentially reinnervated by fast and slow nerve fibres respectively. Although some investigators concluded that muscles showed no preference for their original nerve (Weiss & Hoag 1946; Bernstein & Guth 1961; Miledi & Stefani 1969), experiments in which a fast muscle was reinnervated while having a choice between fast and slow nerve fibres indicated that fast muscle appeared to show a preference for fast nerve (Hoh 1975).

Cross-innervation experiments demonstrated that adult mammalian muscle can readily be innervated by foreign nerves (Buller, Eccles & Eccles 1960; Yellin 1967) and some muscle properties may be altered under the influence of the nerve. However in cross-innervation the muscle had only the choice between incorrect innervation or none at all. A simpler and more natural situation exists where a slow and a fast muscle are reinnervated by their own original common nerve branch. In this case the opportunity for "correct" fast and slow nerve-muscle choices exists on an equal footing with "incorrect" choices, and the situation mimics that occurring during normal nerve repair after injury. If nerves and muscles have identities, biochemical or otherwise, these have the opportunity to be expressed in correct matching in this situation.

The fast lateral gastrocnemius (LG) and slow soleus muscles are innervated in the rat and cat by a common nerve branch (LGS) which enters the LG and innervates it. The remaining nerve fibres then pass through LG to innervate soleus. Cutting the LGS nerve and allowing it to reinnervate the muscles presents the fast LG and slow soleus with a limited choice of axons from their own nerve for reinnervation.

Since the definition by Sherrington (1929) of the motor unit as the functional unit of muscle, whole muscles can be characterized not only by their contractile properties, but also by means of a description of their motor unit population (Close 1965, 1967; Burke et al 1973; Kugelberg 1973; Edgerton et al 1973). As there is normally a high correlation between motor unit contractile properties (and therefore between motor unit types) and the histochemical profile of muscle fibres (Stein & Padykula 1969; Burke et al 1970, 1973), the histochemical profile can also serve to further characterize individual muscles. In order to provide a more rigorous testing of the question of nerve-muscle specificity, the simpler reinnervation paradigm described above was used to allow a fast and a slow muscle to be reinnervated by their own nerve containing both their own and "foreign" axons. The muscles and their motor units were characterized by their contractile and histochemical properties both before and after reinnervation to determine more exactly the nature of the changes occurring in the muscles of the mature mammal after nerve injury. In addition, it was hoped that the degree to which the neuron controls muscle properties might be clarified by comparison of contractile, motor unit and histochemical properties before and after reinnervation.

Specific matching of fast and slow nerve and muscle fibres was not evident during reinnervation. Fast and slow nerve fibres were equally successful in both fast and slow muscle and after reinnervation both muscles contained the same proportion of fast and slow fibres. Nevertheless the two muscles did not become identical. Each muscle retained some of its original characteristics and they therefore remained different in some respects. The slow muscle was slower than the fast muscle after reinnervation although it was faster than a control slow muscle. Similarly, although the fast muscle was slower than control fast muscle it did not become as slow as either the control or the reinnervated slow muscle. The relaxation phase of the fast muscle and the contraction phase of the slow muscle were most affected. Changes in the contraction and relaxation phases of muscle contraction can be attributed to an altered proportion of motor units in reinnervated muscle and the altered motor unit composition is reflected in the altered shape of the twitch contraction record.

This chapter reports the results of the physiological study of the contractile characteristics of the control and reinnervated muscles, and of their motor unit populations. The histochemical study and the comparison of histochemical and motor unit properties will be reported in the next chapter.

2.2.2 Methods

The lateral gastrocnemius-soleus (LGS) nerve was cut before its entry into the lateral gastrocnemius (LG) muscle in 20 male and female Sprague-Dawley rats (175 to 200 grams) under Nembutal anaesthesia (60 mg/kg) and aseptic conditions. The nerve was sewn to the dorsal surface of LG . After four to fourteen months the animals, (now weighing 300 to 500 grams) were again anaesthetised with an initial dose of Ketamine (100 mg/kg) followed by Nembutal (20 mg/kg) intraperitoneally. Anaesthesia was maintained with intravenous Nembutal (20 mg/kg) as necessary and the blood pressure was monitored through an arterial cannula. Both hind legs were prepared for observation by denervation of all muscles except the LG and soleus, and the tendons of these two muscles were tied separately with surgical Mersilene (guage 0). A laminectomy was performed and the ventral roots of L_4 and L_5 were separated from the cord and prepared for stimulation by division into filaments. Filaments were subdivided until a single α-motor axon supplying a single motor unit in either LG or soleus was isolated. Stimulation at low (near threshold) voltage produced and all-or-none response which did not change when the voltage was increased to 4 or 5x threshold. The EMG also remained unchanged at increased voltage, confirming activity of a single motor unit.

The animal was placed in a prone position on a heating pad on a steel plate and the distal femur and calcaneus were secured with metal pins. The tendons of LG and Soleus were freed and the mersilene thread on each tied to force-displacement transducers (Grass FTO3C or FTO3B). The skin around the incisions of the spinal cord and the legs was drawn up and a paraffin pool kept around the spinal cord and the leg muscles. Spinal and rectal temperatures were maintained at 36-38°C. The contraction time found for the rat soleus muscle is considerably longer than the contraction time reported by Close (1967). In our preparation, although the spinal cord and rectal temperatures of the rat remained at 37°C, the leg muscles were at 28-30°C. The Q_{10} for the contraction time of muscle is ~2.5 (Stein, Gordon & Shriver 1982). A temperature correction brings our values for whole muscle into agreement with their findings. The range of motor unit contraction times and tensions agrees with those reported by Close (1967) and Andrews & Part (1972), who reported a range of 20-45 msec for motor unit twitch contraction time. The electromyogram (EMG) of the muscles was recorded by two fine

(75 μm) silver wires inserted into the belly of the LG and soleus muscles. The length of each muscle was adjusted to give maximum twitch force in response to a single square wave pulse of 0.01 msec duration and amplitude 2 times threshold applied to the nerve. One hind limb in eight control animals of comparable age and weight were similarly prepared for study of the motor unit population of their LG and Sol muscles.

The twitch and tetanic contractions of the whole muscles in response to maximal stimulation of the sciatic nerve were recorded in control animals and in the experimental and the control contralateral leg of the experimental animals. The L_4 and L_5 ventral roots on the side of the spinal cord supplying the muscles of interest were divided into fine filaments containing only one α-motor axon to allow stimulation of single motor units.

The twitch force and EMG, after amplification, were displayed on a Tektronix storage oscilloscope and digitized simultaneously by an LSI-11 computer (Digital Equipment Corp., Marlboro, MA). The twitch and tetanic contractions were averaged on-line and the data was stored directly on computer discs. The contractile characteristics were determined from the stored data shown in figure 2.1, as follows:

1. The contraction time (CT)is the time from the beginning of the rise of force to peak force of a single isometric twitch at the optimum muscle length;

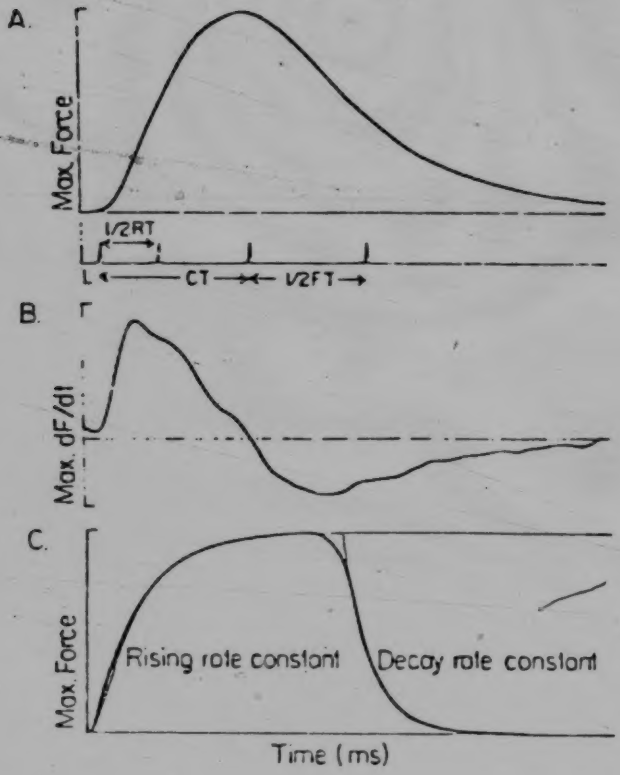


Figure 2.1 Isometric force was recorded from lateral gastrocnemius and soleus muscles in response to stimulation of single motor axons in the ventral roots or maximal stimulation of the motor nerve. The following parameters were automatically computed from averaged records: (A) twitch peak force (mN), contraction time (CT), half-contraction time (1/2 CT), and half-relaxation time (1/2 FT) (ms); (B) from the first differential of the twitch force trace - the normalized maximum rate of rise of force (s -1), and (C) from the tetanic contraction the peak tetanic force, the rising rate constant and the decay rate constant (sec -1) (Stein, Gordon & Shriver 1982). The time scale in (C) is 2x that in (A) and (B). The data shown are from a normal lateral gastrocnemius muscle.

2. The maximum force (F) is the peak amplitude of the force trace of a single isometric twitch at the optimum muscle length;
3. The half rise time (1/2 RT) is the time from the onset of the rise of force for the isometric twitch to rise to 50% of the maximum force;
4. The half fall-time (1/2 FT) is the time required for the twitch tension to decay from its peak to half its maximum value;
5. The maximum rate of rise of tension during a twitch contraction is determined from the peak of the first differential of the twitch curve, normalized by division by the maximum twitch tension (units = seconds -1);
6. The rising rate constant (RRC) obtained by fitting an exponential of the rising phase of the tetanic contraction;
7. The decay rate constant (DRC) obtained by fitting an exponential to a segment of the decay of the tetanus or the twitch;
8. The maximum tetanic tension was defined as the maximum tension produced in response to a train of 20 pulses given at a inter-stimulus interval of one-third of the muscle contraction time (Stein, Gordon & Shriver 1982).

The protocol used for whole muscle was described earlier by Gordon & Stein (1982). Briefly, force and EMG were recorded in response to stimuli of 1, 5 and 20 pulses applied at 2x threshold voltage to the sciatic nerve or to the L_4 and L_5

nerve roots. In order to examine the population of motor units, individual units were examined by division of ventral roots into filaments until stimulation of a filament produced a single all-or-none twitch response, which remained the same when the voltage was kept near threshold. Increasing the voltage to 4 or 5x threshold did not recruit other motor units as indicated by no increase in the force and no change in the shape of the EMG. Force and EMG in response to i) 1 pulse, ii) 5 pulses, iii) 20 pulses - an unfused tetanus was obtained by stimulation by 13-20 pulses at an interstimulus interval of 1.25x the muscle (or motor unit) contraction time. iv) 1 pulse 10 sec. after the tetanus to determine the presence of post-tetanic potentiation or depression, v) fatigue test - stimulation by 13 pulses at an inter-stimulus interval of 25 msec (40 Hz) once per second for two minutes to determine the fatiguability of the muscle fibres (Burke et al 1973). The tetanic contraction was recorded at time 0, 1 and 2 minutes (see figure 2.7). vi) 1 pulse. On completion of the physiological study the control and experimental muscles were removed from the animal, weighed and fast frozen in iso-pentane in liquid nitrogen for histochemical study of the muscle fibres. The details of the histochemical methods and the results will be presented in the next chapter.

2.2.3 Results

2.2.3.1 Contractile Properties of Whole Muscle

Typical records of the force development of the control and reinnervated muscles in response to maximal stimulation of the sciatic nerve are shown in Figure 2.2. Figure 2.2 (A) compares the time course of the twitch contractions of the fast LG and and slow soleus muscles. The control muscles are clearly distinguished from each other by the duration of the twitch contraction, the rate of rise of force and by the rate of relaxation. The time course of the twitch contractions of LG and soleus muscles reinnervated by nerve fibres in the cut and sutured LGS nerve are compared in figure 2.2 (B). The duration of the contraction of the reinnervated soleus is considerably reduced. Although there is some indication that the relaxation phase of the LG twitch is prolonged, this change appears small.

Twitch and tetanic contractions of control and reinnervated muscles are compared directly on the same axes for LG in figure 2.3 (A) and soleus in figure 2.3 (B). Reinnervated muscles exerted less force than the controls, reinnervated LG having an average of 45% of the force of control LG and reinnervated soleus having an average of 61% of the force of control muscles. In order to make a direct comparison of the time course of the twitch and tetanic contractions, the forces were

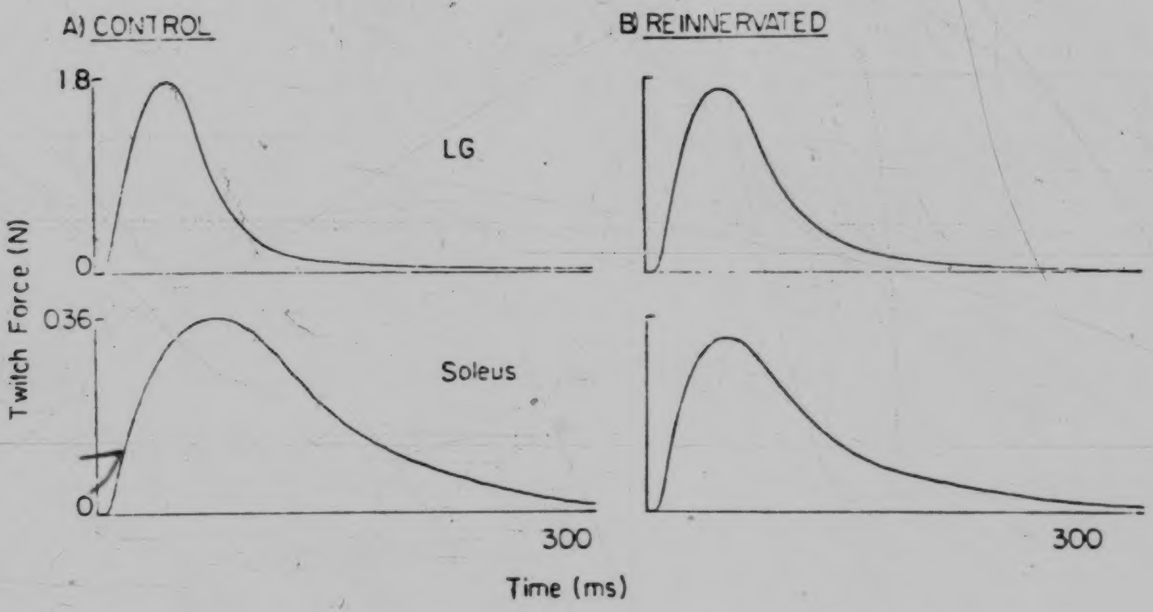


Figure 2.2 Twitch contractions of control LG and soleus muscles are shown on the left and compared with the twitch of reinnervated muscles on the right. The duration of the twitch of control soleus is much longer than that of control LG muscle. After reinnervation the duration of the soleus twitch is reduced and that of the LG is somewhat prolonged.

Figure 2.3 The twitch and tetanic contractions of control and reinnervated LG and soleus muscles are plotted on the same axes. The twitch of control and reinnervated LG is on the left in (A) and the LG tetani are on the right. Control and reinnervated soleus twitch is on the left in (B) and the tetani on the right. The force exerted by reinnervated muscles is less than controls. In order to compare the time course of the twitch and tetanic contractions of control and reinnervated muscles the forces were normalized and the twitch contractions plotted on the same axes on the left in (C) and the tetanic contractions plotted on the same axes on the right. As seen on the left in (C), the rising phase of the twitch contractions of the reinnervated LG and soleus are the same as that of control LG suggesting that both reinnervated muscles have become fast. Both muscles also show some change in the relaxation phase. The rising phase of the tetanic contractions shown on the right in (C) seems to indicate that both muscles are becoming intermediate in speed and similar to each other.

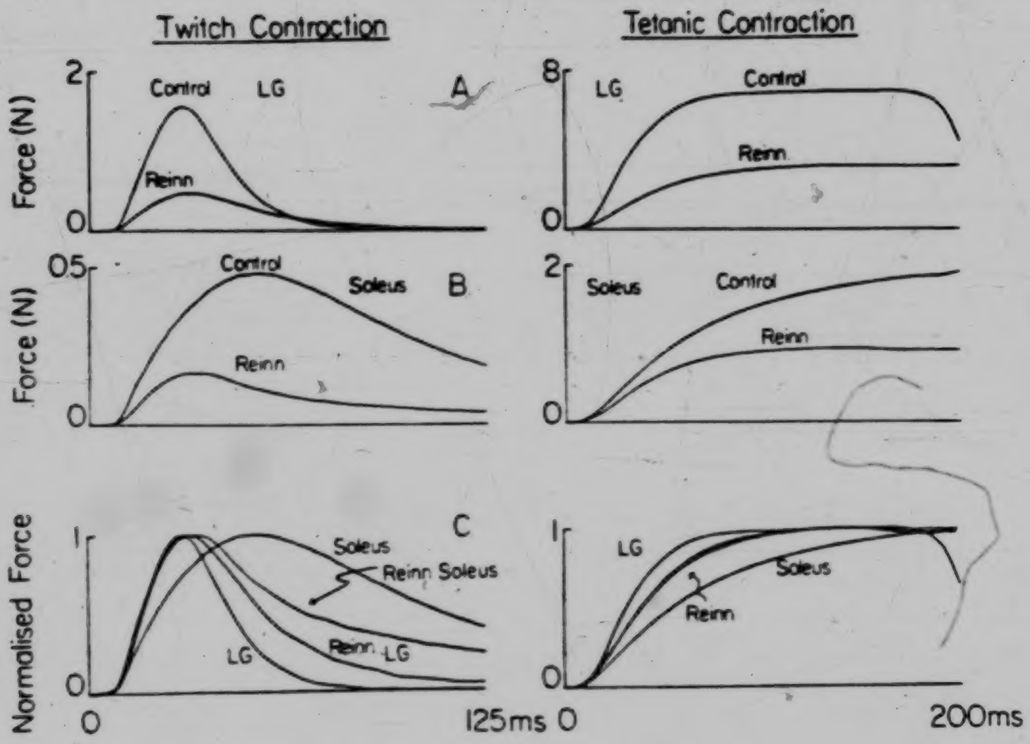

Twitch Contraction
Tetanic Contraction
Force (N)
2
Control
LG
A
Reinn
0
8
LG
Control
Reinn
0
Force (N)
0.5
Control
Soleus
B
Reinn
0
2
Soleus
Control
Reinn
0
Normalised Force
1
C
Soleus
Reinn Soleus
LG
Reinn LG
0
1
LG
Soleus
Reinn
0
0
125ms
0
200ms

Fig. 2.3

normalized and contractions of control and reinnervated muscles were plotted on the same axes in figure 2.3 (C).

Examination of the twitch contractions of the control and reinnervated muscles on the left of figure 2.3 (C) shows that the reinnervated LG develops tension as rapidly as the control LG muscle. but it relaxes more slowly. The rising phase of the twitch contraction of the reinnervated soleus muscle resembles that of the control (and reinnervated) LG muscle and is quite distinct from that of the control soleus muscle. The relaxation phase of the reinnervated soleus muscle resembles that of the reinnervated LG in that both are becoming intermediate with respect to control muscles. The rapid development of tension of both reinnervated muscles suggests that both reinnervated LG and soleus muscles have become fast contracting, perhaps implying that both are innervated by fast motor neurons. However, the relaxation phase of the twitch of the reinnervated LG appears prolonged and similar to that of reinnervated soleus suggesting that both muscles are becoming intermediate and are similar to each other. The rate of development of tension for the tetanic contractions, shown on the right of figure 2.3 (C) also suggests that the time course of the contractions of both muscles is becoming intermediate betweeen that of either a predominantly fast or slow muscle.

Further examination of the characteristics of twitch and tetanic contractions shows that the falling phases of all contractions are well fitted by a simple exponential as shown in figure 2.4 (A, B) (cf methods, Stein et al 1982), giving a rate constant for the falling phases of twitch and tetanic contractions. The rising phase of tetanic contractions can be similarly fitted wit[illegible] an exponential to give a rate constant for the development of tension. The maximum rate of increase of the force was calculated as the normalized rate of change of force per second. Comparison of control and reinnervated muscles indicates a significant change in the rising and falling phases of the tetanic contractions: the rate of development of force and the rate of decay of force of tetanic contractions fall in reinnervated LG while these rates increase in reinnervated soleus. Note however that the rate of decay of tension in tetanic contractions of soleus muscles is not very different to that of the contralateral controls when the simple exponentials fitted to the decay of force are compared (figure 2.4).

Parameters of the time course of contractions of reinnervated and control muscles were calculated as described above and in the methods. The contraction time and the half-rise time were significantly shorter and the calculated parameters of the rate of development of tension - the rate constant and the maximum rate of

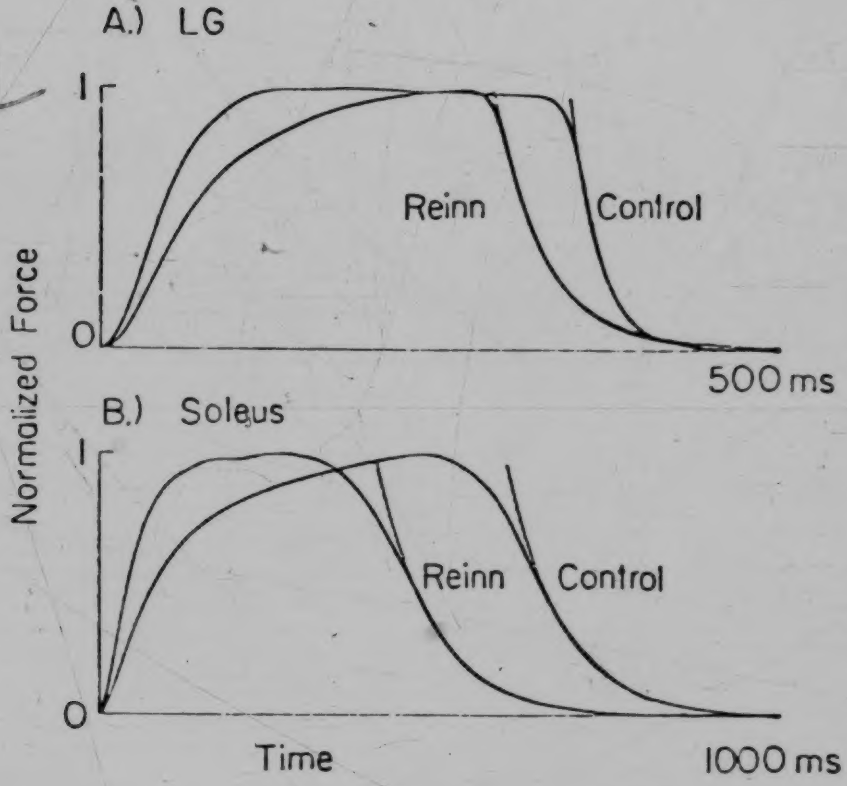


Figure 2.4 Tetanic contractions of control and reinnervated LG in (A) and soleus in (B) are plotted on the same axes with the force normalized. A simple exponential can be fitted to the decay of tension. The change in the rate of relaxation and in the rate of development of tension of each muscle after reinnervation can be seen and can be calculated from the fitted exponential.

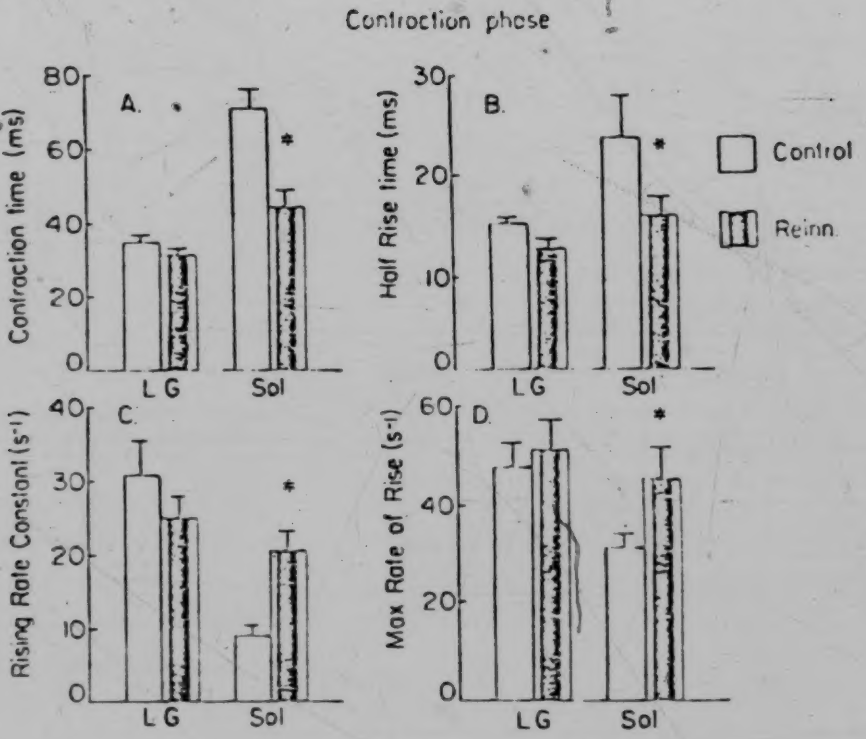


Figure 2.5 The contraction time (A), half-rise time (B), the calculated maximum rate of rise of force (C) and rising rate constant (D) - parameters of the rising phase of the contractions are compared for LG and soleus. Each of the measurements of contraction time and calculated values for the rate of development of tension is significantly increased for soleus muscle (by a t-test for differences between means, $p< 0.01$) and has become similar to values in control LG. There is little change in the twitch contraction time and the rate of development of tension in the LG muscle after reinnervation. (Asterisks denote significant differences between reinnervated and control muscles).

development of tension were significantly faster than normal for soleus muscle after reinnervation as determined by a t-test for differences between means ($p < 0.05$) and became similar to all LG muscles in each case. The bar histogram of figure 2.5 shows the significant change in the rate of development of tension of reinnervated soleus as indicated by the contraction time, half rise time and the calculated rising rate constant and the maximum normalized rate of rise of tension while those values are not significantly changed from controls for the reinnervated LG muscle.

Changes in the relaxation phase in reinnervated muscles are shown in the bar histogram of figure 2.6. The half-relaxation time of reinnervated LG increased significantly (by a t-test for differences between means $p < 0.05$) and the decay rate constant was significantly reduced ($p < 0.05$) Although reinnervated soleus appears to show a trend in the opposite direction the differences are not siginificant as shown in figure 2.6. LG muscles, in contrast to the soleus muscles, showed a more pronounced change in the relaxation phase toward values found for slow muscle.

Thus, the contraction phase of the slow twitch soleus muscle was most affected by reinnervation by the severed common LGS nerve while the relaxation phase was most affected in the reinnervated LG muscles. Comparison of the contraction phase of reinnervated muscles

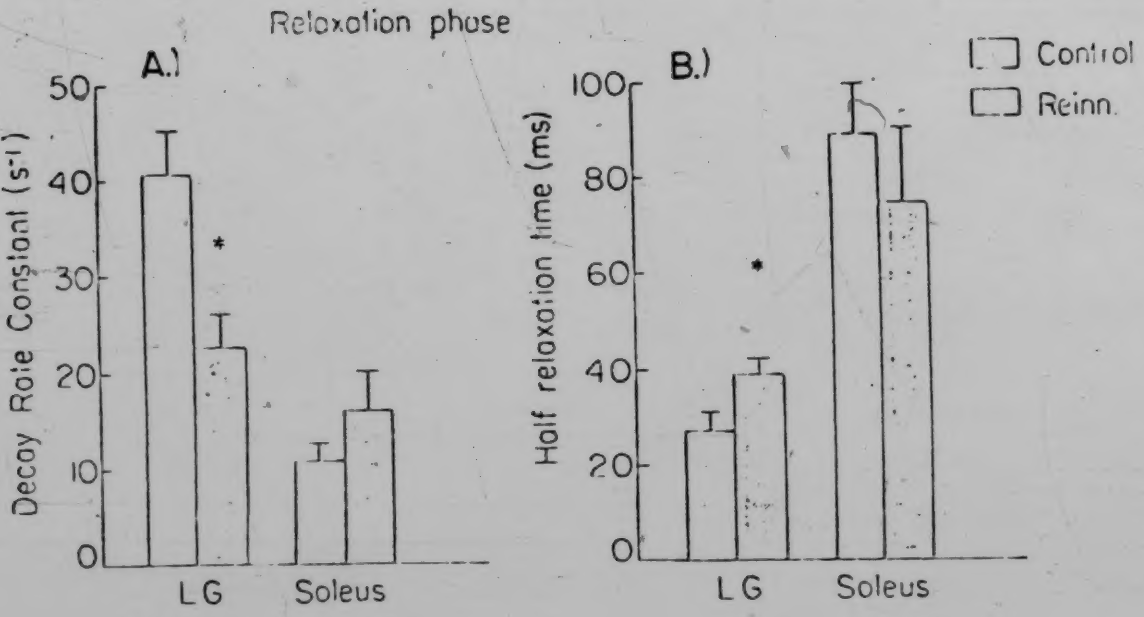


Figure 2.6 The time course of the relaxation phase of contractions, described by the half relaxation time (A) and the decay rate constant (B) are compared for control and reinnervated muscles. The half-relaxation time is significantly longer and the decay rate constant is significantly reduced (by a t-test for differences between means, $p< 0.05$) in the LG after reinnervation while the trend in the opposite direction in soleus muscle is not siginificant. (Asterisks denote significant differences between reinnervated and control muscles).

suggests that fast nerve fibres may have preferentially reinnervated both muscles since both reinnervated muscles resemble control fast muscle. However, it is clear that the relaxation rate of LG is slowed implying some reinnervation by slow nerve fibres. Similarly, the intermediate relaxation rate of soleus indicates some reinnervation by slow fibres.

2.2.3.2 Classification of Motor Units in Control and Reinnervated Muscles

A possible explanation of the changes in the time course of the twitch and tetanic contractions is that fast fibres tend to dominate the rise of force while the slow fibres present limit the rate of relaxation of the whole muscle. If this is the case, the prolonged relaxation time in LG implies reinnervation by more slow fibres than normal and the increase in the rate of rise of force during contraction in soleus would be due to reinnervation by more fast nerve fibres than normal. To determine directly the proportion of slow and fast nerve fibres which had reinnervated the LG and Soleus, the L_4 and L_5 ventral roots were split into fine filaments containing only one α-motor axon to either LG or soleus to stimulate single motor units (as described in the methods).

One hundred and nine control and 61 experimental motor units were studied. Eighty-seven control and 43 experimental motor units were studied in the LG muscles and 22 control and 18 experimental motor units in the soleus muscles.

Motor units in the control rat lateral gastrocnemius-soleus were classified into four major types -fast-fatiguable (FF), fast-intermediate (FI), fast, fatigue-resistant (FR) and slow (S), using contraction time and fatigue index as the criteria. The fatigue index (FI) is "the ratio of maximum tension produced (by the muscle or motor unit) after two minutes of stimulation to the tension output during the first tetanus in the standard sequence" (Burke et al 1973). Units having a fatigue index of 0.75 or less had relatively brief contraction times (< 30 ms) and were classified as fast-fatiguable (FI=0.25 or less), or fast-intermediate (FI=0.25 to 0.75) on the basis of their susceptibility to fatigue as reported earlier by Fleshman et al (1983) for cat MG and Kernell et al (1983) for cat peroneal muscles.

The range of contraction times of motor units showing fatigue-resistance (FI >0.75) included both fast and very slow motor units. The calculated decay rate constant plotted as a function of contraction time of motor units in control and reinnervated muscle shows a negative correlation in both control and reinnervated muscle as shown in figure 2.7. The relaxation rate for all fatigue resistant motor units shows considerable overlap.

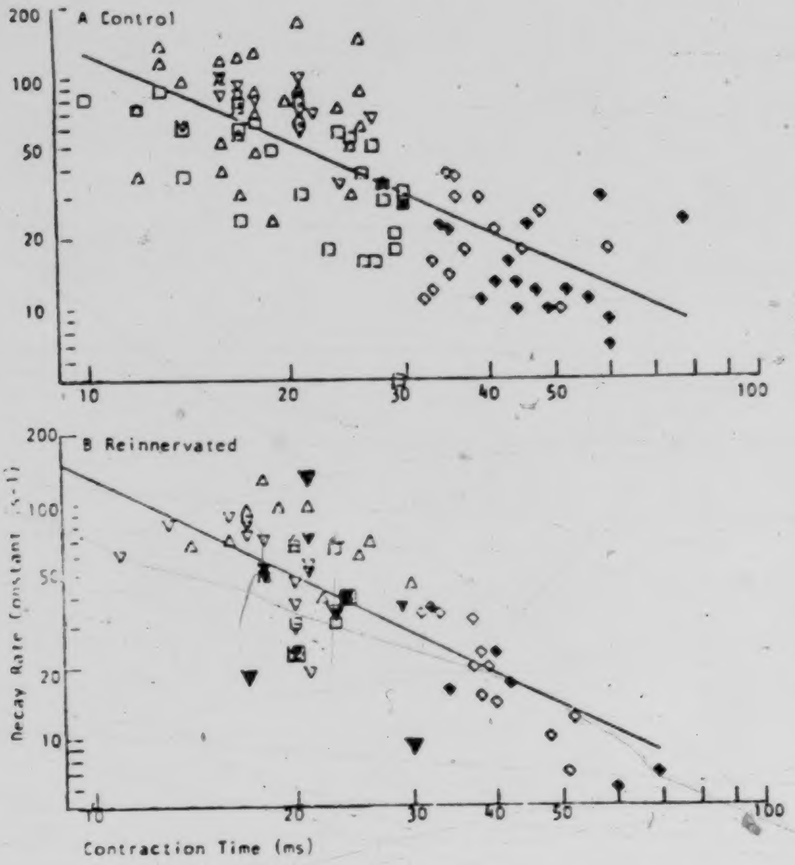


Figure 2.7 The decay rate constant plotted as function of contraction time on a double-logarithmic scale shows a negative correlation for control and reinnervated muscles. It can be seen that in both control and reinnervated muscle the relaxation rate for fatigue resistant motor units shows considerable overlap. The slope of the regression line fitted to the data of control and reinnervated motor units is significantly different from 0 (control motor units= -1.41 ± 0.11, ρ=0.78 ($p < 0.01$) and reinnervated units the slope = -1.41 ± 0.77, ρ=0.77 ($p < 0.01$)). Symbols are FF (Δ), FI (∇), FR (■) and S (<>). Open symbols are units in DG and filled symbols are motor units in soleus.

However, distinct differences could be demonstrated between slow and fast fatigue-resistant motor units using the stimulus paradigm described in the methods for the fatigue test as shown in figure 2.8. Stimulation by 13 pulses at 40 Hz produced an unfused tetanus with fast motor units, and a fused or nearly fused tetanus with slower units. Fatiguing fast units did not show "sag" as described by Burke et al (1973) during the first 5-10 pulses but showed a drop in tension by one minute during the fatigue test. Burke's criteria included the use of the presence or absence of "sag" in an unfused tetanus for classification of motor units. Sag was described as "an early tension maximum during the first six or seven stimuli with a subsequent slight decline or 'sag' to a lower plateau". Conversely, a motor unit showing no sag has "a tension envelope described by the peaks of individual components which rose monotonically to reach a stable plateau at long tetanus durations". Recent reports indicated that use of the sag criterion as originally defined by Burke et al (1973) did not provide consistent discrimination between motor unit types (Chan et al 1982) and allowed for an overlap of twitch contraction time between sagging and non-sagging units (Kernell et al 1983). It also resulted in a less powerful component of slow motor units relative to the proportion of histochemically slow fibres. Kernell et al (1983) reported the method of classification by

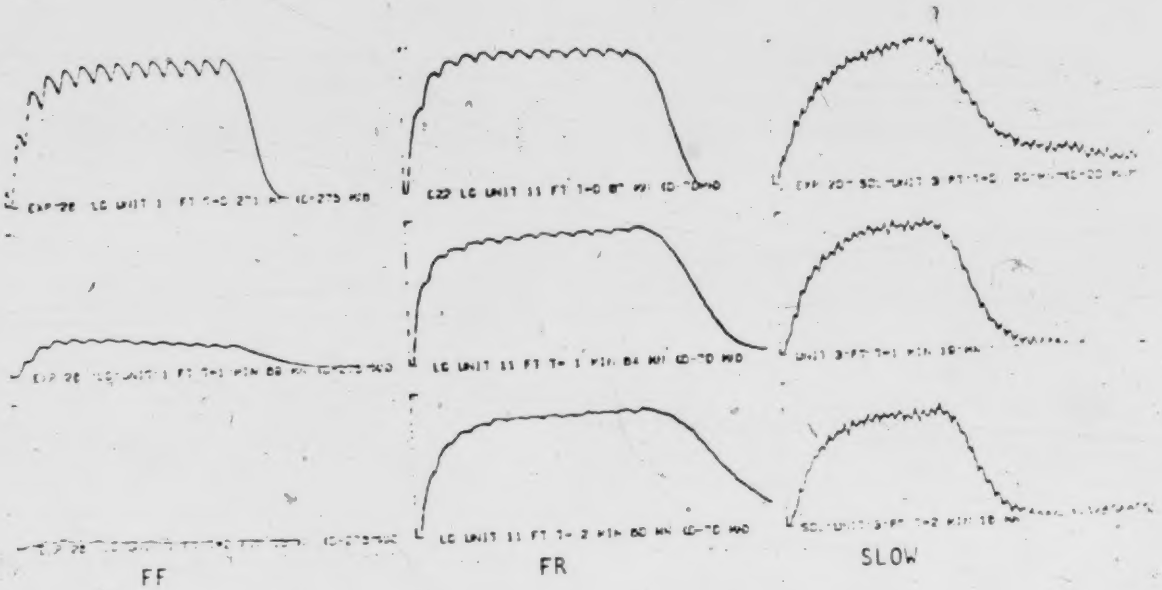


Figure 2.6 Typical records of motor unit tetanic contractions elicited once per second for two minutes during the fatigue test are shown at times of 0, 1 and 2 minutes for typical FF, FR and S motor units. The fast units developed an unfused tetanus when stimulated for 13 pulses at 40 Hz once per second. The relaxation rate slows during the repetitive train of stimuli causing the tetanic contractions of the fast units to become less 'rippled' with time. The slow units developed a nearly fused contraction which shows 'rippling' due to the greater amplification of the smaller forces of slow units. The fatiguing units developed 'sag' in the force trace by the 1 minute record. The tension of the slow units rose continuously during the 13 pulses throughout the fatigue test. The contraction time of the FF unit on the left was 18 ms, the FR in the centre 29 ms and the slow unit on the right 42 ms.

contraction time and fatigue-sensitivity to be "at least as valid as with the sag criterion". Slow units consistently had a rising tension profile throughout the two minute fatigue test. Figure 2.8 shows typical force traces obtained from FF, FR and S motor units at the start of and after 1 minute and 2 minutes stimulation during a fatigue test, demonstrating the characteristic differences between slow and fast fatigue-resistant motor units.

Using this classification there was a clear division between fast and slow control LGS motor units on the basis of contraction time. The contraction times of fast motor units were 30 ms or less in control muscles while those of slow motor units were greater than 30 ms. Therefore the motor units in reinnervated muscle were classified as fast or slow according to their contraction time (greater or less than 30 ms) and fast motor units further classified according to their fatigue index. The tension profile during the fatigue test and therefore the "sag" of reinnervated motor units remained consistent with the classification as described for control motor units for 95% of the reinnervated motor units. It was of interest however that three reinnervated slow motor units (two in soleus and one in LG) with long contraction times showed fatigue (FI=0.31).

2.2.3.3 The Contractile Speed of Motor Units in Control and Reinnervated Muscles

The distribution of the contractile speed of the motor units sampled in control and reinnervated muscles is shown in the histogram in figure 2.9. The range of contraction times for the soleus motor units is similar to that reported by Close (1967) and Kugelberg (1973).

The time course of the twitch contractions and the rate of rise of force (fig. 2.10) and the rate of fall of force (Fig. 2.11) during twitch and tetanic contractions did not change significantly after reinnervation for the population of motor units studied. (In both these figures the single FI motor unit in control soleus and the single FF motor unit in reinnervated soleus are included without error bars for comparison). The contraction time and half-rise time of twitch contractions in all fast motor units in reinnervated LG muscles are the same as those in control LG muscles and are significantly different from the slow motor units in both control and reinnervated muscles. Soleus fast motor units after reinnervation are similarly not significantly different from soleus control motor units after reinnervation although the contraction times showed higher variability. Units were consistently slower than the corresponding type of unit in LG ($p< 0.05$)(fig.2.10 (A and B)). The maximum rate of development of twitch tension (fig. 2.10 C) is the same

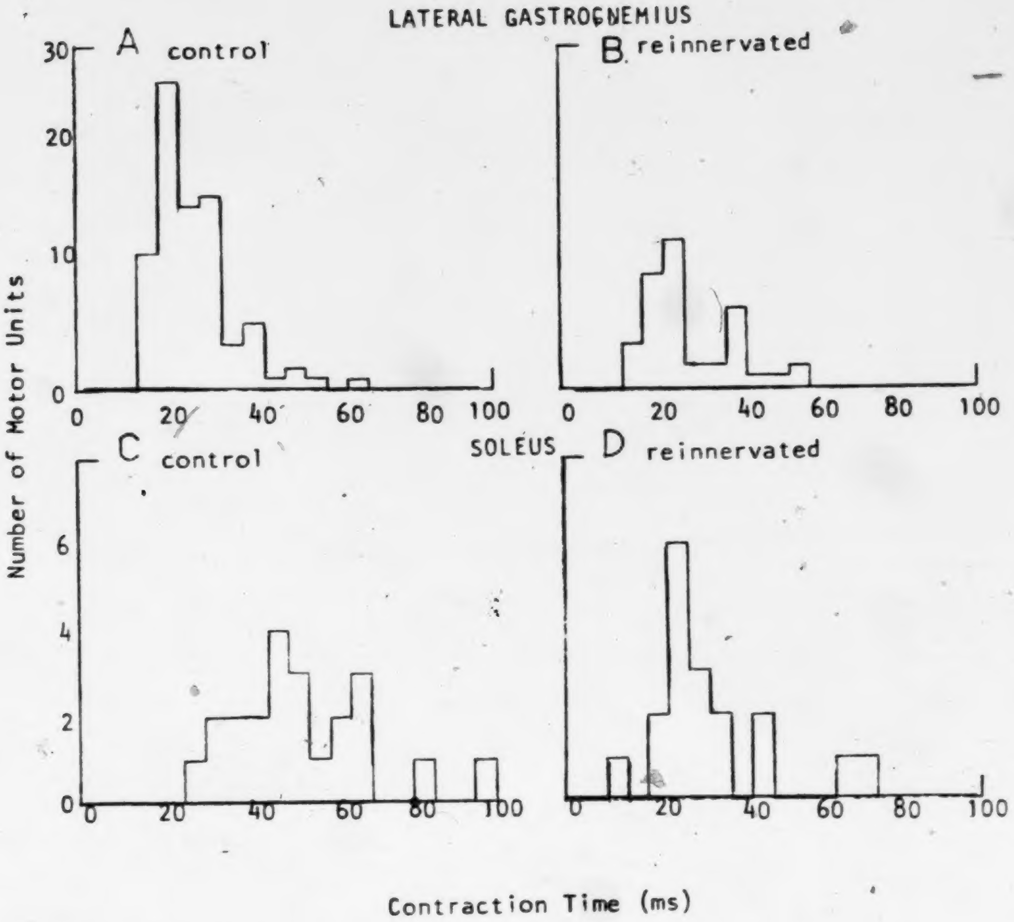
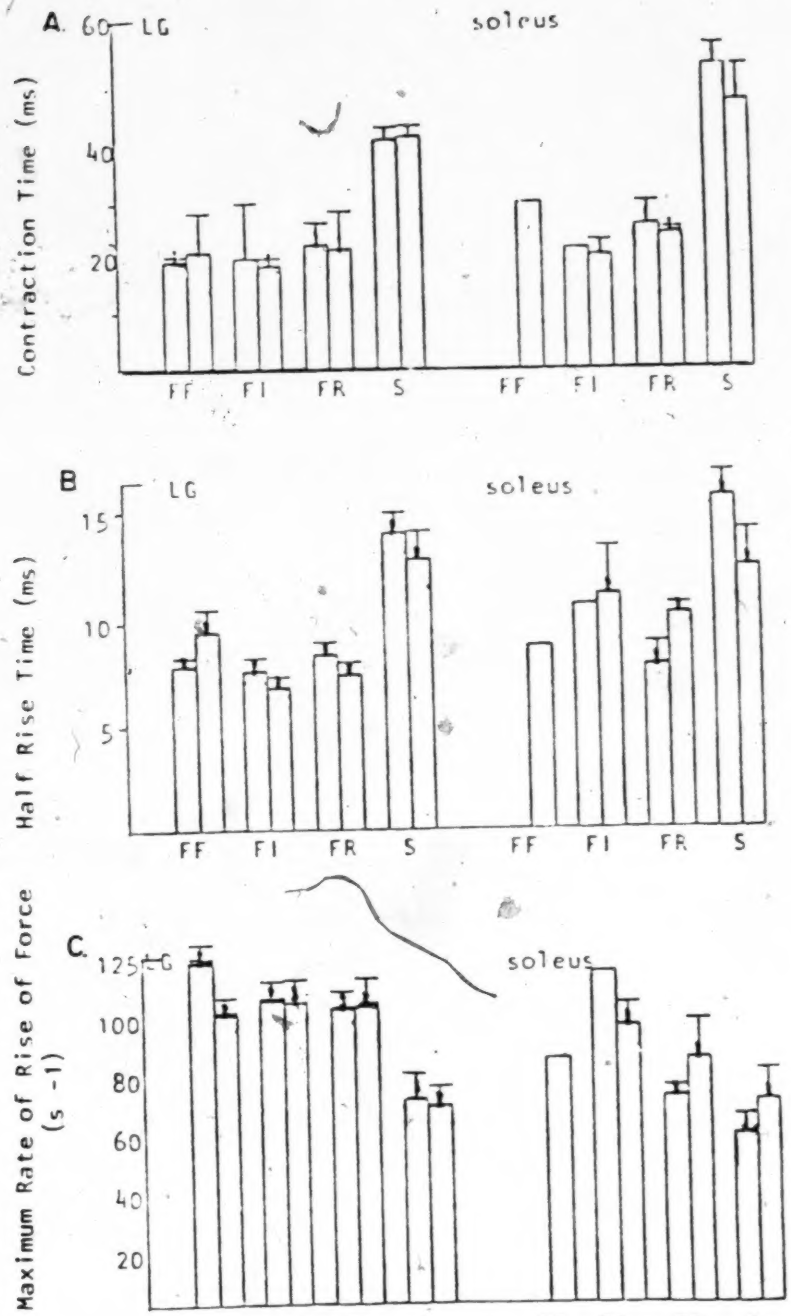


Figure 2.9 The histograms show the numbers and the contraction times of the motor units studied. Control lateral gastrocnemius contains primarily fast motor units (CT <30 ms) as shown in (A) while after reinnervation (B) there are relatively more slow motor units in the muscle (see table 2.1.) Control soleus (C) contains primarily motor units with long contraction times. After reinnervation a population of fast contracting motor units were found in soleus muscle (D). Note that the unit scale (on the y-axis) for soleus motor units is larger than that for lateral gastrocnemius.

Figure 2.10 Means and standard errors of (A) the contraction time (ms) (B) the half-rise time (ms) and (C) the maximum rate of rise of force (s-1) for the development of tension are shown for for FF, FI, FR and S motor units in control and reinnervated LG on the left and control and reinnervated soleus on the right. Values for a single experimental FF and control FI unit in soleus are included without error bars for comparison. There is no significant difference between control and reinnervated muscle motor units for the time measurements and rate parameters determined by a t-test for differences between means ($p < 0.01$).


A.
60
LG
soleus
40
20
Contraction Time (ms)
FF
FI
FR
S
FF
FI
FR
S
B
LG
soleus
15
10
5
Half Rise Time (ms)
FF
FI
FR
S
FF
FI
FR
S
C.
125
LG
soleus
100
80
60
40
20
Maximum Rate of Rise of Force
(s -1)
FF
FI
FR
S
FF
FI
FR
S

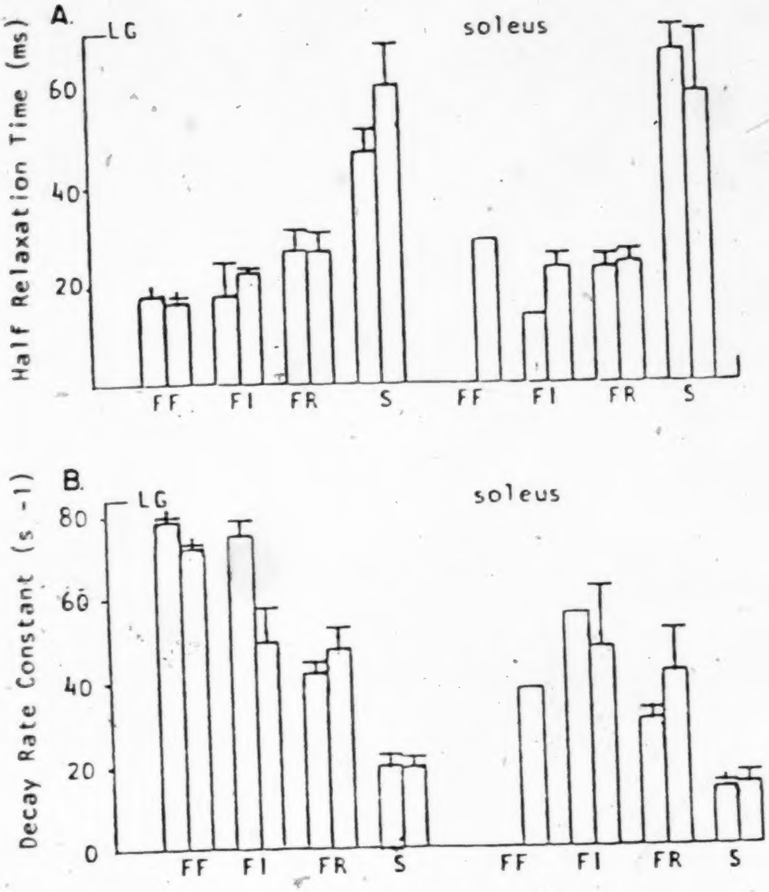


Figure 2.11 Means and standard errors of (A) the half-relaxation time (ms) and (B) the decay rate constant (s-1) for FF, FI, FR and S motor units in control and reinnervated LG on the left and control and reinnervated soleus on the right. There is no significant difference in relaxation speed between control and reinnervated motor units of each type as determined by a t-test for differences between means ($p < 0.05$).

for all LG control fast motor units and significantly lower only in reinnervated slow units, which became similar to reinnervated soleus slow motor units. Control soleus motor units have a lower rate of tension development in general than LG control motor units. The reinnervated FR motor units in soleus develop tension as rapidly as LG motor units.

With respect to the relaxation phase, the motor units show a gradation of speeds according to motor unit type. (Relaxation times of LG FF and FR motor units are significantly different as well as being significantly faster than slow units). Control slow motor units in soleus are significantly slower to relax than LG slow motor units. There is no significant change in the relaxation time of motor units of either muscle after reinnervation (fig.2.11 A). and there are no significant changes in the decay rate constant for motor unit types after reinnervation. (fig 2.11 B). (Significance was determined in all cases by a t-test for differences between means). Reinnervated motor units in soleus showed more variability in all the above measurements. This is due in part to difficulty in obtaining measurements that were as accurate for very small motor units as those for the larger motor units in reinnervated LG.

Thus, the alterations observed in the time course of the development and decay of tension of whole muscle during twitch and tetanic contractions are not due to corresponding changes in the speed of contraction and relaxation of fast and slow motor units in the reinnervated muscles. The range of contractile speed (fig. 2.9) and the means of a number of different parameters of contractile speed of motor unit types in reinnervated muscle are typical of those of normally innervated muscle (fig. 2.10 and 2.11). The alteration in whole muscle contraction speed can be attributed to a change in the proportions of motor unit types as shown below.

2.2.3.4 Proportions of Motor Units in Control and Reinnervated Muscle

The proportions of motor units studied in the control and reinnervated muscles are shown in table 2.1. The majority of the motor units in the control LG were fast contracting, and only 17% slow motor units were identified. The control Soleus contained 14% fast fatigue-resistant and 82% slow motor units. There was one fast intermediate and no fast fatiguable motor units found in control soleus.

The most striking aspect of the motor unit composition of the reinnervated muscles was the change in proportion of the different types of units. Where the proportions of slow muscle fibres were grossly different in control muscles (17% for LG and 80% for Soleus as shown in table 2.1), they were very similar in

	CONTROL				REINNERVATED			
	FF	FI	FR	S	FF	FI	FR	S
LG	40	15	28	17	35	21	16	28
Sol		4	14	82	6	39	22	33

Table 2.1 The percentage of FF, FI, FR and S motor units identified in control and reinnervated lateral gastrocnemius and soleus muscles. The proportion of slow motor units is very similar in both reinnervated muscles which suggests that reinnervation by fast and slow motor neurons was non-selective. Neither the fast or the slow muscle was preferentially reinnervated by its original type of nerve.

reinnervated muscles (28% in LG and 33% in Soleus). Reinnervated LG retained a population of fast-contracting motor units with contraction times in the range of 10 to 22 msec, but had far fewer motor units with intermediate contraction times (figure 2.9 B). The reinnervated soleus differed from its control in having acquired a large percentage (67%) of large motor units with fast contraction times between 18 and 30 msec which included some classified as FR and FI motor units, one FF unit and fewer slow motor units (figure 2.9 D) whereas the control muscle contained nearly entirely slow and fatigue-resistant fast motor units (figure 2.9 C).

2.2.4 Discussion

"Random" Reinnervation

A striking finding in this study is that after reinnervation both the originally fast LG muscle and the originally slow soleus muscle contain close to the same proportion (~30%) of slow motor units. Significantly more slow nerve fibres reinnervate the LG and significantly more fast nerve fibres reinnervate the soleus than in the control muscle. The muscles were equally well reinnervated (see the next chapter) indicating that neither the fast nor the slow muscle was refractory to reinnervation by the LGS nerve fibres. If the muscles had not shown preference for their former type of nerve supply, then the proportions of fast and slow motor units in each muscle should be predicted by the proportions of each type of axon in the reinnervating nerve. A simple calculation, shown in table 2.2, uses the muscle twitch forces and the proportions of motor units contained in them to estimate the actual number of motor units in each muscle. An estimate of 27 slow and 38 fast fibres in the LGS nerve predicts that, if reinnervated equally, both the LG and soleus muscles will contain 35% slow motor units. Our data show the reinnervated LG muscle contains 28% slow motor units and reinnervated soleus contains 33% slow motor units. This is consistent with the conclusion that reinnervation was "random" or non-selective with respect to the proportion of successful fast and slow nerve fibres in each muscle. The fast and slow nerve fibres appear equally successful in both muscles and synaptogenesis on denervated muscle fibres has occurred regardless of the original type of the muscle fibre. Although it has been shown that regenerating axons have a strong tendency to return to their original terminations at the end-plate of muscles (Bennett et al 1973; Sanes et al 1980) it appears that denervated fast and slow muscle fibres do not necessarily attract their former type of nerve.

The figures indicate that there was a reduction in the absolute proportion of slow nerve fibres (from 36% to 30%). This is contradictory to the suggestion of Lewis et al (1982) that slow motor neuron fibres appear to be more successful than fast in reinnervation. Our apparent loss of

	Lateral Gastroc	Soleus	LGS nerve
Muscle force (mN)	1650 ± 173 (8)	291 ± 30 (3)	-
Motor unit force (mN)	40 ± 4.3 (74)	12 ± 1.3 (15)	-
No. of motor units Muscle force/unit force	41	24	65
Proportion of slow units (%)	17	82	35
Total no. of slow units	7	20	27
Total no. of fast units	34	4	38

Table 2.2 The number of fast and slow motor neurons in the common LGS nerve can be estimated by determining the number of motor units in LG and soleus muscles indirectly by division of the total muscle force by the motor unit force. The proportion of slow and fast motor units was obtained experimentally by classification of the units according to their contraction time and fatigue-sensitivity. This calculation permits an estimate of the number of fast and slow motor neurons. Column 3 shows that the LGS nerve contains 65% fast nerve fibres and 35% slow nerve fibres.

slow nerve fibres could be the result of sampling error since our population of experimental motor units was not large but our finding of a normal distribution of motor unit sizes in the reinnervated muscles (as described in the next chapter) suggests that this was not a probable source of error.

Although earlier experiments designed to demonstrate nerve-muscle specificity did present denervated muscles with an array of axons including their own by nerve-nerve suture of the sciatic nerve or its major branches, (Bernstein & Guth 1961; Yellin 1967; Miledi & Stefani 1969), or by conduction of two major nerve trunks through an arterial splice back to their former destination (Weiss & Hoag 1946) these procedures were at a considerable distance from the muscles, where trophic influence of the muscle apparently does not influence course of growth of the nerve sprouts. The complications created when the dis-ordered axon sprouts entered the endoneurial tubes of whichever portion of the distal stump they first encountered (Cajal 1928; Guttman & Sanders 1943) may well have precluded the self-reinnervation of muscles that it had been hoped to demonstrate. Once having entered what may well be an inappropriate endoneurial tube, axons are constrained within it (Brown & Hopkins 1981) and will grow down that pathway to what may be an incorrect target muscle. Physical obstruction within the muscles of either of two nerves may have similarly compromised the conclusions reached from attempts to allow "competitive" reinnervation to occur (Ellsberg 1917; Hoh 1975; Riley 1978). The experimental design of cross-innervation between a slow and a fast muscle (Eccles et al 1960; Close 1967; Dubowitz 1969) precluded the selection of "self" muscle fibres by nerve although a number of reports of cross-innervated muscle have indicated contamination of the muscles by self reinnervation (Dubowitz 1967; Bagust et al 1981). Therefore cross-innervation studies in which there was very little possibility of correct reinnervation, and uncertainty regarding the exact reinnervation of the muscles have previously left the question of nerve-muscle specificity unresolved.

The strategy used in the present study of reinnervation of applying both the fast LG axons and the slow soleus axons directly back to their original muscles in their own common nerve eliminated the possibility of gross misdirection of the axons during regeneration while preserving some of the choices that were available to the nerves during embryogenesis. The axons had the same opportunity as they had developmentally to respond to environmental cues within the muscle and to be directed by contact guidance along their former endoneurial tubes, and possibly to match fast and slow nerve and muscle fibres according to their biochemical identity. Nerve re-growth was directed by contact guidance from the connective tissues of both nerve and muscle (as will be described in the next chapter) but the result was that the slow and fast fibres became markedly separated and each type of fibre formed motor units that were confined within muscle compartments. It has been shown that "type-grouping" occurs when regenerating nerve fibres form motor units by reinnervating adjacent muscle fibres (Karpati and Engel 1968a). Even under these circumstances the proportions of fast and slow nerve fibres in reinnervated muscle was the same as in the nerve branch supplying the muscle.

The Trophic Influence of the Nerve on Muscle

Cross-innervation experiments have consistently led to the conclusion that the nerve can alter the contractile speed of the muscle, (Buller et al 1960; Close 1967; Dubowitz 1967) but the finding that the conversion of some muscle contractile properties towards those of the type of muscle formerly innervated by the nerve was incomplete, especially for slow muscle, suggested a limitation of the ability of the nerve to control muscle properties (Robbins, Karpati & Engel 1969; Edgerton et al 1980). Hypotheses presented to explain the intermediate contractile properties of whole muscle have been that either all fibres have undergone partial change to a similar degree and are all intermediate in type (Buller & Lewis 1965; Close 1969; Robbins, Karpati & Engel 1969; Sreter et al 1975; Hoh et al 1980), or that reinnervated muscle contained a mixture of fast and slow fibres (Bagust et al 1973; Chan et al 1982). Findings of this study provide the first clear evidence to support the latter suggestion. The new nerve supply determines the contractile properties of individual muscle fibres so that the properties of each motor unit type were similar to control in reinnervated motor units as shown in figures 2.10 and 2.11. There was no evidence that reinnervated motor units acquired intermediate contractile speed characteristics, but rather that the alterations in contractile properties of whole muscle are due to the altered proportions of each type of motor unit in the muscle. These proportions will dictate the overall time characteristics of the muscle twitch as we have shown for the reinnervated LG and soleus muscles. The fast motor units in a muscle will cause a fast rate of rise of twitch tension and the slow motor units will extend the duration of the twitch and control the rate of relaxation of the muscle.

The method of classification of motor units provides good discrimination between types of units. The characteristics of the relaxation phase may prove more useful than the contraction time for differentiation between types of motor units. It has been demonstrated that half-relaxation time or decay rate constant are directly related to the contraction time but provide for a better distinction between types of units than the contraction time (Kernell et al 1983).

Changes in whole muscle properties after reinnervation are inadequately described using the contraction time alone. Much more information is provided by examination of the characteristics of the rising and falling phases of the twitch and tetanic contractions, and by examination of the motor unit population. The proportions of fast and slow motor units in a muscle are responsible for the contractile characteristics.

2.3 LIMITATION OF THE TROPHIC CONTROL OF MUSCLE PROPERTIES BY THE NERVE

2.3.1 Introduction

A number of recent reports in cross-innervation studies have indicated that there are limitations to the extent of the neural control of muscle properties. Conversion of some of the contractile properties of muscles under the influence of a foreign nerve includes alterations of the myosin isoenzymes (Sreter et al 1975; 1980; Bagust et al 1981; Srihari et al 1981; Gauthier et al 1983), and alterations in the levels of activity of oxidative enzymes and the mitochondrial content of the fibres. Recent research has suggested that there may be a dissociation between control of these two types of enzymes (Sreter et al 1975) and that some properties may be controlled primarily within the muscle and are thus resistant to neural control (Edgerton et al 1980; Burke et al 1982; Chan et al 1982; Gauthier et al 1983). In order to further clarify which mechanisms are influenced by the nerve, this study examined the contractile and histochemical properties of a fast and a slow muscle and of their motor unit population following reinnervation by the muscle nerve branch common to both muscles. Comparison was made of the changes in each of these types of muscle properties after reinnervation.

The proportions of slow and fast types of motor units found in reinnervated muscle (see the previous chapter) showed that the nerve axons had not preferentially selected their own original muscles or type of muscle fibre on reinnervation but that reinnervation of both muscles by their common nerve was random with respect to the degree of success of axons of either fast or slow nerves. Both the originally fast muscle and the originally slow muscle contained the same proportion of slow motor units after reinnervation, and that proportion was predicted by the ratio of fast and slow motor nerve fibres in the reinnervating nerve. The contractile speed of each muscle was altered due to the alteration in the motor unit population observed in each muscle but the parameters of contractile speed of individual motor unit types remained unaltered. The contractile speed of individual muscle fibres had been controlled by the nerve. Results to be presented in this chapter show that there was an alteration of the histochemical profile of the muscles which corresponds only in part to the change in the motor unit population. The originally slow muscle, soleus, retained a disproportionately large percentage of oxidative fibres containing acid-stable ATPase after reinnervation compared to the motor unit population demonstrated physiologically, while muscle fibres in the fast LG (lateral gastrocnemius) muscle showed the high degree of correspondence between histochemical profile and motor unit population that was normally seen in control muscle. The fast LG muscle readily adapted its histochemical properties with altered reinnervation but soleus, in contrast, although readily accepting innervation by fast motor neurons showed limited adaptability of its oxidative profile and ATPase enzymes as demonstrated by histochemical methods.

Preliminary data from examination of reinnervated cat hind-limb muscles (Gordon, Stein & Gillespie 1980) pointed to similar conclusions but indicated that it was necessary to examine the entire reinnervated muscle in order to determine accurately the extent of the changes observed since many of the changes were focal, affecting only a part of the muscle. To do this the smaller muscles of rats were used and cross-sections of the entire muscle were examined histochemically.

2.3.2 Methods

The common lateral-gastrocnemius soleus (LGS) nerve was cut before its entry into the lateral gastrocnemius (LG) in the hind limb of rats, denervating the LG and soleus muscles, and sewn to the dorsal surface of the LG muscle under aseptic conditions. After 4-14 months the contractile properties of the muscles and their motor units were recorded in an acute experiment as described in the previous chapter.

After completion of the study of the contractile properties of the muscles and individual motor units, the muscles and their contralateral controls were removed, cut into 3-4 mm lengths and mounted on small pieces of cork with OCT (Tissue Tek, Miles Laboratories Inc.). These samples were fast-frozen either in isopentane cooled by liquid nitrogen to -160°C or directly in liquid nitrogen after being coated with talcum powder (Moline & Glenner 1964). Sections were stored at -80°C as necessary prior to being cut and stained. For staining, muscle samples were transferred to a cryostat and allowed to warm to a temperature of -20°C. The corks were mounted on the cryostat chucks and serial sections of muscle cut 8-10 μm thick. The sections were picked up on glass coverslips, air dried at room temperature and stained for oxidative enzyme activity: reduced nicotinamide adenine dinucleotide diaphorase (NADH-D)(Novikoff, Shin & Drucker 1961), glycolytic activity: menadione-linked α-glycerophosphate (m-α-GPD) (Pearse 1968) and ATPase activity after acid (pH 3.9-4.3) and alkaline (pH 10.5) pre-incubation according to the method of Guth & Samaha (1970).

Methods used were as follows:

1. NADH-D: Sections were incubated for 30 min. at pH 7.4 at 37°C in 0.2M MOPS (morpholinopropanesulfonic acid) buffer containing 8 mg NADH-D (reduced nicotinamide adenine dinucleotide) and 10 mg NBT (nitro-blue tetrazolium) per 10 ml.
2. m-α-GPD (menadione-linked α-glycerophosphate dehydrogenase): Sections were incubated for 45 min. at

37°C in 0.2M Tris buffer at pH 7.4 with 4mg menadione, 10 mg NBT, 40 mg α-glycerophosphate.

3. ATPase: Sections were pre-incubated in either acid or alkaline media as follows:
   a. acid preincubation: Glacial acetic acid was diluted 0.3 ml per 100 ml (final volume) in 18mM $CaCl_2$ and the pH was adjusted to two or three pH levels as necessary with 5N KOH before dilution to the final volume. The optimal pH for rat muscle was found to be in the range of 3.9 to 4.3.
   b. alkaline preincubation: 18mM $CaCl_2$ in 100mM 2-amino-2-methyl-1-propanol was adjusted to pH 10.4 or 10.5 using 1 or 6N HCl (back-titrating with KOH if necessary).
4. After acid pre-incubation for 3-5 minutes or alkaline preincubation for 10-12 minutes slides were washed in 2 x 1 min. changes of 100 mM Tris (pH 7.8) containing 18 mM $CaCl_2$. Incubation for 30 min. at 37°C in a solution of 2.7 mM ATP (Adenosine triphosphate - Sigma Chemical Corp.), 50 mM KCl, 18 mM $CaCl_2$ in 100 mM 2-amino-2-methyl-1-propanol buffer at pH 9.4 was followed by 3 x 30 sec rinses in 1% $CaCl_2$ solution, incubation for 3 min. in 2% $CoCl_2$, rinse in 4 x 30 sec changes of distilled water, and incubation for 3 min. in 1% (v/v) ammonium sulphide. The sections were then washed in running tap water for 3 min. (Guth & Samaha 1970).

The number of fibres staining positively for oxidative and glycolytic enzymes and for fast and slow myosin ATPase were counted in serial sections and the fibres classified as f.g. - fast, glycolytic, f.o.g. - fast, oxidative glycolytic and s. - slow as described on page 53 and as shown in table 2.3.

2.3.3 Results

2.3.3.1 The Extent of Recovery of the Muscles

Both the LG and soleus muscles were successfully reinnervated in all the experimental animals, and it was noted that the nerve to soleus travelled from the deep surface of the LG in a single trunk as it normally does, indicating that the regenerating nerve had made use of its original connective tissue sheath for guidance. The nerve fibres may have entered the LG via the original epineurial sheath. The pattern of enzyme staining in the reinnervated muscles indicates that the connective tissue of the muscle confined regenerating axons within compartments of the muscle. The relative weight, number of muscle fibres and force in reinnervated muscles as compared with control contralateral muscles is shown in table 2.4. The extent of recovery of force and weight are consistent with findings of previous studies which showed that muscles do not fully recover to their pre-operative levels of force after nerve-muscle suture in contrast to the greater recovery shown after

Staining Intensity	f.g.	f.o.g.	s
ATPase alkaline pre-incubated pH 10.4	dark	dark-mediun	light
acid pre-incubated pH 3.9-4.3	intermediate	light	dark
NADH-D	light	dark	medium
α-GP	dark	dark	light

Table 2.3. Staining intensity of muscle fibres when stained for myosin ATPase and oxidative (NADH) and glycolytic (α-GP) enzyme activity. Fibre types are f.g. - fast, oxidative-glycolytic, and s - slow as described on page 58 and 122. Classification according to Peter et al. (1972) and Burke et al. (1973).

Ratio Experimental/Control	Muscle Weight	No. of fibres	Twitch Force (mN)
LG	0.68 ± 0.04 (21)	0.68 ± 1.26 (4)	0.45 ± 0.14 (10)
Soleus	0.62 ± 0.05 (21)	0.82 ± 0.61 (3)	0.61 ± 0.14 (7)

Table 2.4 The extent of recovery of the muscles. The ratio of experimental versus control values of the muscle weights, number of fibres and twitch force shows good reinnervation of both muscles. Although soleus is reinnervated after the LG it is reinnervated as well or better than LG and recovers a greater proportion of force.

nerve-nerve suture (Gordon & Stein 1982). It is interesting to note that while the recovery of muscle weight in reinnervated soleus was equal to that of reinnervated LG, the relative number of muscle fibres and the twitch force was higher. This indicates that motor units in soleus had become larger as confirmed by motor unit average twitch force and the innervation ratio.

Both muscles appeared to have readily accepted reinnervation by the axons in the mixed muscle nerve. Some reinnervated muscles were seen to include "bands" of very small fibres which were atrophic (Dubowitz & Pearse 1960), and were therefore not counted as part of the fibre population in the histochemical study. Reinnervated LG has 69% of its original number of muscle fibres supplied by 90% of its original number of nerve fibres while soleus had 81% of its original muscle fibres and only 42% of its original number of of nerve fibres. Since atrophying muscle fibres were noted (figure 2.16) this suggests either failure of contact or mis-matching between nerve and muscle and rejection of inappropriate synapses in the LG muscle which had the first opportunity for reinnervation. Fewer nerve fibres were available to reinnervate soleus muscle and these have done so by means of enlarged motor units. Some atrophying muscle fibres were also seen in reinnervated soleus.

Application of the calucation of table 2.2 to reinnervated muscle implies that the proportion of slow nerve fibres had decreased to 52% of their original number fibres and the fast nerve fibres had decreased to 81% of their original complement while the muscles contained 70-80% of their original fibres after reinnervation (table 2.4). This suggests that the slow nerve fibres have been less successful than fast nerve fibres in terms of their ability to reinnervate muscle. Those slow fibres which were successful were able to reinnervate an increased number of muscle fibres.

2.3.3.2 Properties of the Motor Units

The Force and the Contraction Times of the Motor Units

Motor units were classified as fast and slow according to their fatiguability and contraction time as described in detail in the previous chapter. Consistent with earlier data on reinnervated motor units in cat LGS muscles (Gordon & Stein 1982), there is a significant inverse linear relationship between the size of motor units as indicated by their tetanic tension (the tension after 5 pulses).[3] and their contraction time in the reinnervated LGS muscles (figure 2.12 B) similar to the normal size relationship (figure 2.12 A),

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[3] Not all motor units were tetanized for 20 pulses since some rapidly fatiguing motor units were already showing a reduction in tension before 20 pulses were delivered to them. These motor units were then 'tetanized' for only 5 pulses at an interval of 0.33 to 0.40 of the twitch contraction time (Stein & Parmiggiani 1979).

Figure 2.12 An inverse linear relationship can be shown on a double-logarithmic plot between motor unit size as indicated by tetanic tension and contraction time for motor units studied in control (A) and reinnervated (B) LGS. (slope of the computed regression line line in (A) - control muscle was -1.1 ± 0.15, ρ=0.58 ($p < 0.01$) and in (B) reinnervated muscle was -0.74 ± 0.27, ρ=0.36 ($p < 0.05$). Largest motor units have the shortest contraction times and smaller units have progressively longer contraction times. Symbols are as in figure 2.7

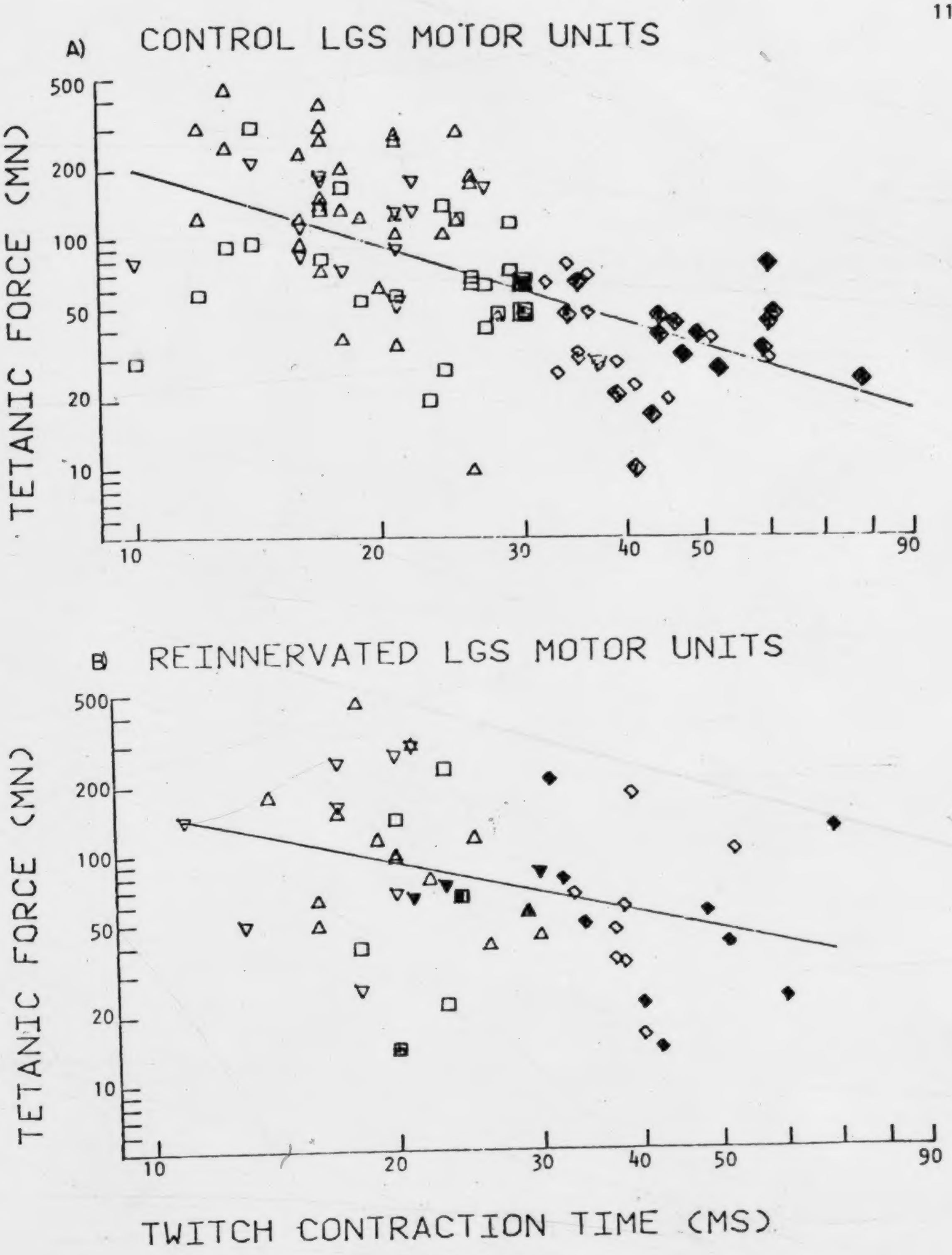

A) CONTROL LGS MOTOR UNITS
TETANIC FORCE (MN)
500
200
100
50
20
10
10
20
30
40
50
90
B) REINNERVATED LGS MOTOR UNITS
TETANIC FORCE (MN)
500
200
100
50
20
10
10
20
30
40
50
90
TWITCH CONTRACTION TIME (MS)

Figure 2.12

although the data points for reinnervated muscle are more scattered. The largest motor units have the shortest contraction times and smaller motor units have longer contraction times. (The slope of the regression line on double-logarithmic axes for motor units in control LG is -1.1 ± 0.15, $\rho=0.58$> ($p< 0.01$) and for the motor units found in reinnervated LG is -0.74 ± 0.27, $\rho=0.36$ ($p< 0.05$).

The average twitch force of motor units in control and reinnervated muscle as shown by the bar histogram of figure 2.13 includes a more limited range of motor unit sizes in the reinnervated muscles than in control muscles. No very large motor units similar to the large FF motor units of control muscle were found after reinnervation. Although the size of the FF and FI motor units were significantly reduced in reinnervated LG the relative sizes of fast motor units are retained with FF units being the largest and FI, FR units being progressively smaller as shown for the control motor units (figure 2.12 and 2.13). The limited range of fast motor unit sizes tends to support the suggestion made by Chan, Edgerton et al (1982) that the size of motor units may be limited, at least for the fast motor units whether or not muscle fibres remain denervated. The average force of all reinnervated FR and S units in both muscles is not significantly different ($p<.05$). The force of the reinnervated motor units in soleus muscle

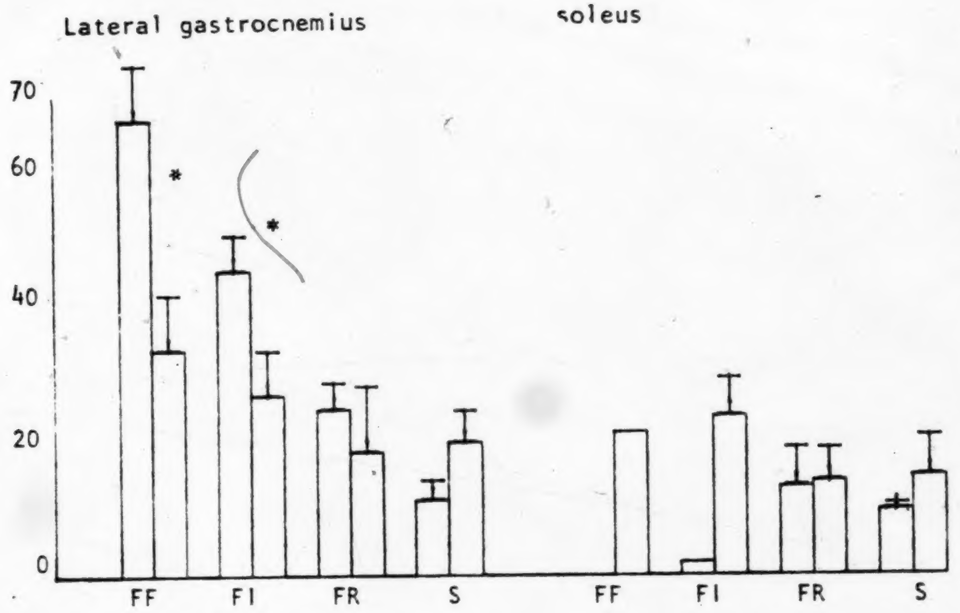


Figure 2.13 The size of each type of motor unit as measured by the average twitch force (mN) in control and reinnervated muscle. In control muscle the FF motor units are the largest and FI, FR and S progressively smaller. While the fast reinnervated motor units show a tendency to return to that relationship the FR and S motor units are not significantly different in size. The average size of FF and FI motor units is significantly reduced in reinnervated muscle. No very large FF motor units such as those in control muscle were found after reinnervation. Values for control motor units are on the left of each pair and reinnervated motor units are on the right. Asterisks denote significant differences between control and reinnervated motor units.

showed greater variability than the other motor units. Many of reinnervated soleus fast motor units were smaller than those normally sustained by the fast motor axons when supplying normal LG muscle. The recovery of force in soleus (table 2.4) can be accounted for by the increased proportion of fast motor units in the reinnervated muscle.

Resistance to Fatigue

The normal relationships found between motor unit size, contraction time and fatigue resistance were retained after reinnervation for fast motor units. These relationships which are found in the normal motor unit population continue to be present in the reinnervated muscles except that, as pointed out above, no very large FF units similar to those in control LG were identified in the reinnervated muscles and many of the fast motor units in reinnervated soleus were small.

The boundaries for fatiguability were less rigid. Two units that were clearly FF according to all other criteria (contraction time, half-rise time, half fall-time and rate constants) had a fatigue index of 0.71 (within the range of FI units). Three slow motor units in reinnervated muscle fatigued (fatigue index ~0.30). Two of these were in soleus and one in LG.

Twitch/Tetanic Ratio

The relationship between the size of the twitch and that of the tetanic tension of the motor units in control muscles is retained after reinnervation The twitch-tetanic ratio of the whole muscles was not significantly different ($p < 0.05$) after reinnervation (control LG 0.29 and reinnervated LG 0.27, and control soleus 0.23 and reinnervated soleus 0.25). The ratio for soleus agrees with that reported earlier by Close (Close 1967; Close & Hoh 1969).

2.3.3.3 Histochemical Properties of Control and Reinnervated LG and Soleus Muscles

Classification of Fibre Types in Normal and Reinnervated Muscle

The fibres in experimental muscles, control contralateral limb muscles of experimental animals and muscles from control animals were classified according to the criteria given by Burke et al (1973) and Peters et al (1972), as previously summarized in table 2.3. Fibres with high glycolytic enzyme activity, low oxidative enzyme activity, and alkali-stable ATPase were classifed f.g. (fast, glycolytic); fibres staining strongly for oxidative activity, intermediate for glycolytic activity and with low ATPase activity after acid pre-incubation were classified f.o.g. (fast, oxidative-glycolytic); and fibres that stained dark for oxidative enzyme activity, light for glycolytic activity and with high ATPase activity after acid pre-incubation were classified s. (slow).

Differences in staining intensities between fibres of the same type (Brooke & Kaiser 1969; Guth & Yellin 1972; Kugelberg 1973) due to the variable levels of activity of oxidative and glycolytic enzymes, may result in up to 15% error in subdivision of the fast fibres into two groups (Reichmann & Pette 1983). Difficulty in classification of fast fibres is greatest in the rat, and it has also been noted (Kugelberg 1973; Burke 1983) that rat soleus muscle is unique in having the f.o.g. fibres stain more intensely for NADH-D activity than the slow fibres. In the cat the slow motor units in soleus are different from the slow motor units in the gastrocnemius (Burke et al 1974). (Since completion of this study Gollnick (1983) has reported a technique which will further subdivide the fast and slow fibres.) Nevertheless the muscle fibres could be satisfactorily classifed into three groups using the combination of four stains (as described in the methods) which allowed for comparison of the histochemical and physiological data. The method was to count the numbers of fibres of each type in each stain of whole muscle cross-sections and calculate the proportions of each. Fibres were compared in serial sections to confirm that the classification by each stain corresponded with the other stains. The large numbers of fibres counted (see table 2.4) allowed for statistically reliable results and it could easily be seen that the pattern of staining of reinnervated muscle was duplicated with all stains except for the oxidative enzyme stain of reinnervated soleus. Interpretation of the data was undertaken with the above-mentioned possible degree of error in mind. The correspondence obtained in our data between motor unit and histochemical fibre types suggest that the fibre classification is reasonably accurate.

Srihari et al (1981) reported conversion of myosin isoenzymes and myosin light chains in the contralateral limb as well as the experimental, and Reichmann et al (1983) found that cross-reinnervation of limb muscles induced changes in the contralateral muscles as well, although less extensive than in the experimental muscles. We found no significant differences in the results in this study between the contralateral leg muscles of the experimental animals and those of control rats, and no significant differences between the group of animals studied and 4-7 months (half) and those studied at 14 months.

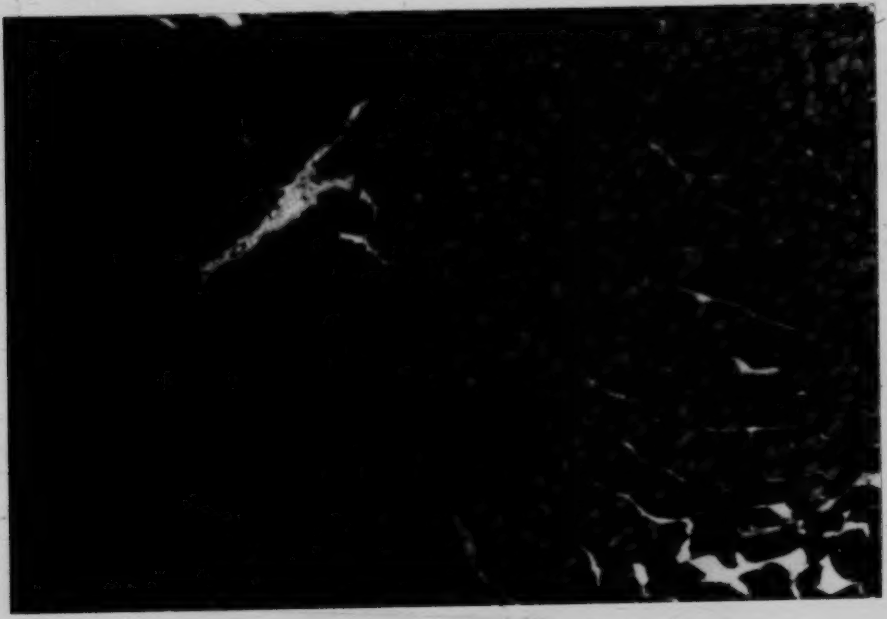
Distribution of Fibre Types in Control and Reinnervated Muscle

Control soleus muscle stained intensely for oxidative enzymes and 20% of its fibres, evenly scattered in a mosaic through the muscle, were very slightly darker with NADH-D. These fibres stained positively (although faintly) for glycolytic activity and positively for alkali-stable ATPase and were thus classified as f.o.g. fibres. The superficial part of the control LG (lateral gastrocnemius) contained only uniformly staining glycolytic fibres with alkali-stable ATPase activity (f.g. fibres) but in the deeper areas, increasing towards the ventro-medial part of the muscle there was a mosaic with increasing proportions of f.o.g. and slow fibres. Control LG is shown in figure 2.14 (A) stained for NADH-D activity, and control soleus is shown in figure 2.14 (B) stained for myosin ATPase activity after acid pre-incubation (pH 4.2). In contrast, figure 2.15 shows typical stains of reinnervated muscles in which, in both LG and soleus muscles, the types of fibres are well separated or "type-grouped". Figure 2.15 (A) shows a reinnervated LG muscle stained for myosin ATPase after acid pre-incubation (pH 4.2) and figure 2.15 (B) shows a reinnervated soleus muscle stained for myosin ATPase activity after acid pre-incubation (pH 4.2).

In the reinnervated LG the fast and slow (oxidative and glycolytic) fibres tended to form bands across the muscle in the dorso-ventral direction. Dorso-ventral bands of fascicles of very small atrophic fibres were seen "compressed" in the belly of reinnervated muscles or along the edge of the muscle, frequently in animals studied within 4-6 months of nerve suture and less often after longer periods of reinnervation. A reinnervated LG muscle with a band of very small atrophic fibres

Figure 2.14 (A) Control LG muscle stained with NADH-D for oxidative enzyme activity. The superficial part of the muscle is entirely f.g. fibres. Oxidative f.o.g. and slow fibres appear in a mosaic in the deeper parts of the muscle. (B) Control soleus stained for myosin ATPase activity after acid pre-incubation (pH 4.2). The fast (f.o.g.) fibres are light and are seen in a mosaic distributed evenly throughout the muscle. (Magnification x26)

A



COLOURED PICTURES
Images en couleur

B

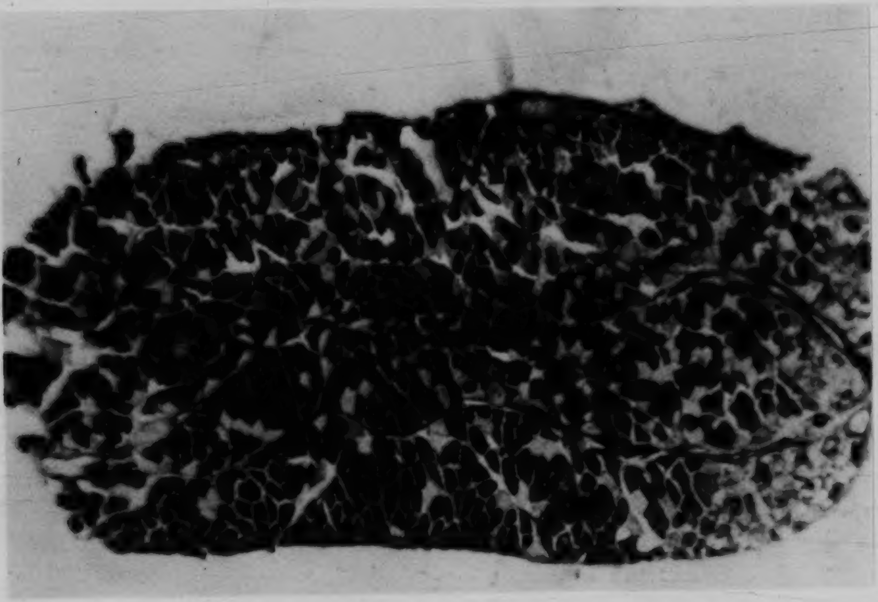


Figure 2.15 (A) Reinnervated LG muscle stained for myosin ATPase activity after acid pre-incubation (pH 4.2). Fast fibres are light. Fast and slow fibres are markedly "type-grouped" or separated. (B) Reinnervated soleus muscle stained for myosin ATPase activity after alkaline pre-incubation (pH 10.4). Fast fibres are dark and are found mainly in one or two clusters. (Magnification x26)

A

COLOURED PICTURES
Images en couleur

B

is shown in figure 2.16. Most of the fast fibres in reinnervated soleus were found in one or two clumps and atrophic fibres were usually seen at the periphery of the muscle or near one end. Fibres of reinnervated soleus stained almost uniformly for oxidative enzymes (NADH-D) but fast and slow fibres could be identified by the stain for glycoclytic activity and myosin ATPase.

2.3.3.4 Comparison of the Histochemical Properties and the Motor Unit Population of Reinnervated Muscle

The proportions of f.o.g, f.g. and s. fibre types for control and reinnervated muscles are shown in a histogram in figure 2.17 and compared with the proportions of FF, FI, FR and S motor unit types before and after reinnervation. (n.b. For purposes of comparison the FI motor units for which there is no separate corresponding fibre type are grouped with the FR units, since they are more likely to be of the same histochemical type, and this group are compared with the histochemically f.o.g. fibres).

The proportion of motor units of each type and the proportions of histochemically identified fibres correspond fairly closely in the control and the reinnervated LG (figure 2.17 A). The reinnervated LG contained significantly more histochemically slow fibres than the LG control muscle (33% vs 9%) and had 28% slow motor units in contrast to 17% in the controls.

COLOURED PICTURES
Images en couleur

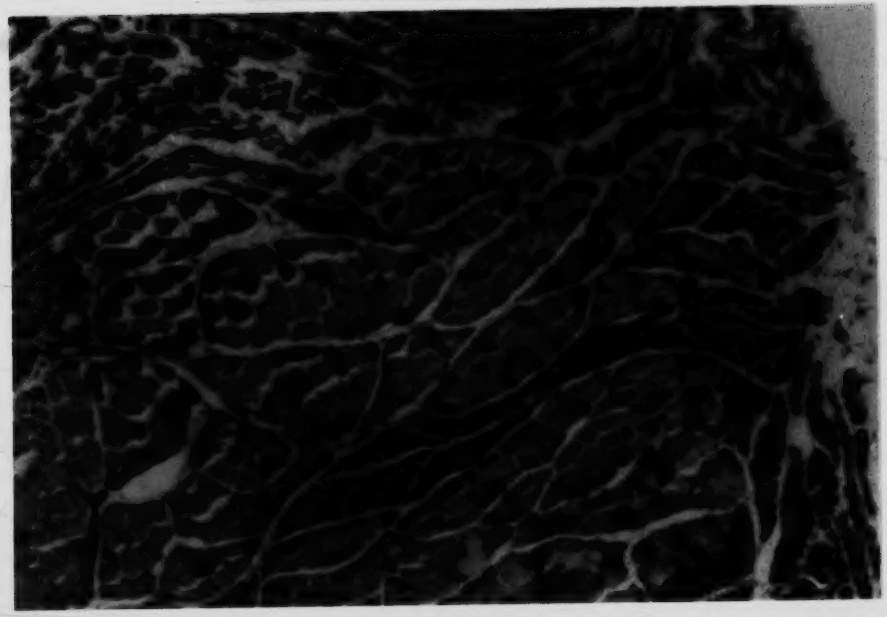


Figure 2.16 A portion of a reinnervated LG muscle stained for myosin ATPase after alkaline pre-incubation (pH 10.4). Bands of very small atrophic fibres are seen compressed in the belly of the muscle. This muscle was studied 5 months after nerve suture. (Magnification x300)

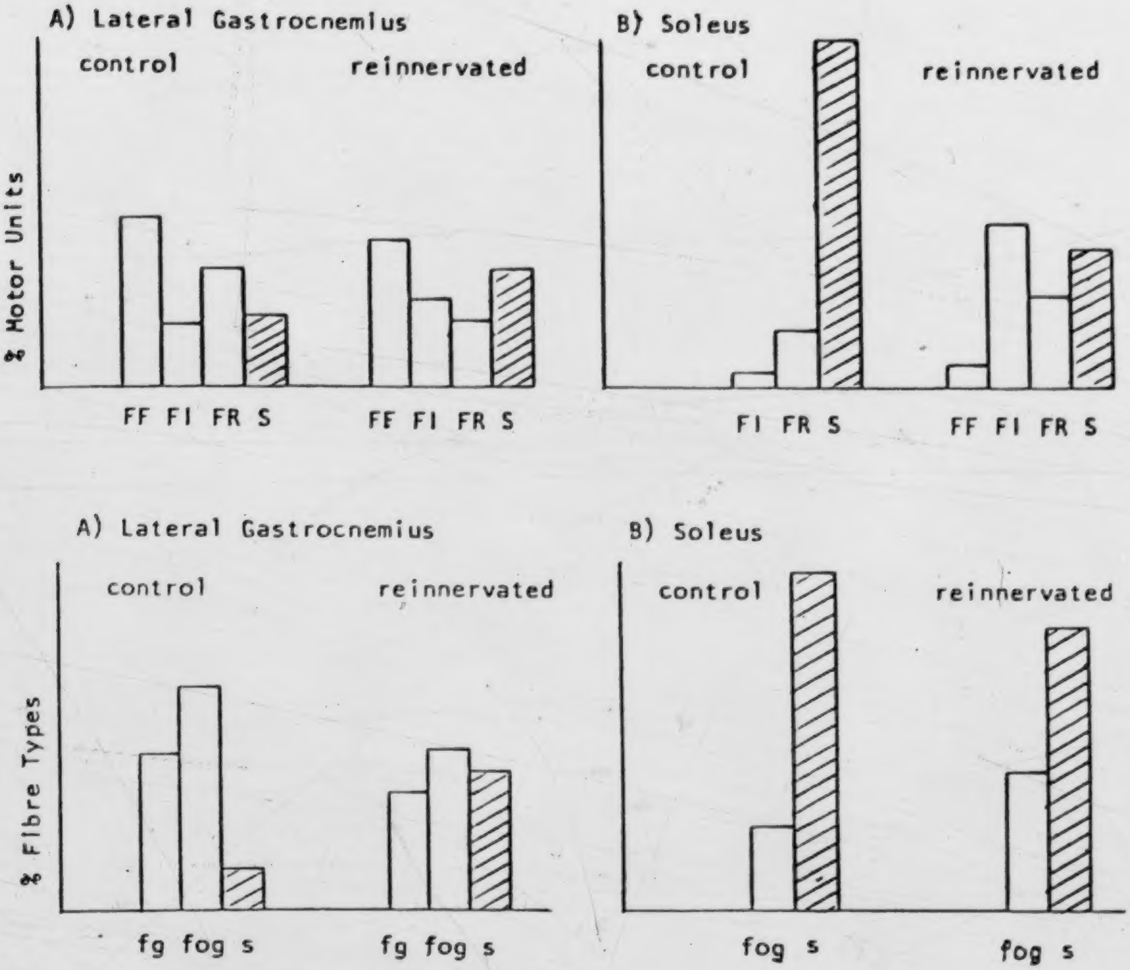


Figure 2.17 The relative proportions of each type of motor unit and each type of histochemically identified fibre type. The proportions of motor units and fibre types correspond in the LG muscle both in the control and reinnervated muscles (A). While the proportions of motor units and fibre types correspond in control soleus muscle as shown in (B) this relationship is not found in the reinnervated muscle.

In striking contrast to the correspondence between the number of slow motor units and the number of histochemically identified slow muscle fibres in the reinnervated LG muscle, there was little correspondence between the high proportion of histochemically slow fibres in reinnervated soleus and the much lower proportion of slow motor units (figure 2.17 B) The high proportion (82%) of slow motor units in control soleus corresponds well to the proportion (80%) of histochemically identified slow (oxidative) fibre types. In contrast the reinnervated soleus retained a high proportion (67%) of oxidative fibres with slow myosin ATPase as identified by the ATPase and α-glycerophosphate stains while the physiological data indicate that reinnervated soleus contained a much lower proportion (33%) of slow motor units than control muscle. (All fibres in reinnervated soleus stained relatively strongly for oxidative enzyme activity so the NADH-D stain was not considered a reliable indicator). The proportions of slow motor units in both LG and soleus after reinnervation were similar and the value is close to the proportion of 35% slow motor axons in the reinnervating nerve. Thus, there appears to be resistance of slow muscles to conversion of their metabolic pathways for energy production under the influence of the nerve, despite the demonstrated conversion of their contractile speed.

2.3.3.5 The Number of Motor Units and the Innervation Ratio (IR) of the LG and Soleus

Using the average twitch force of the whole muscle and the average force of each type of motor unit in each muscle the number of motor units (and therefore motor neurons) supplying the control and reinnervated muscle can be calculated as was shown in table 2.2 of the previous chapter for the control LG. Table 2.5 shows an estimation of the number of motor neurons and the innervation ratio of both control and reinnervated LG and soleus. The innervation ratio is determined by division of the total number of muscle fibres by the number of motor nerve fibres. There are an estimated 41 motor units in the control LG and 24 in the control soleus while reinnervated LG has 37 motor units and reinnervated soleus 10. The value for control soleus is slightly below previous estimates of the number of motor units in soleus of 32 (Zelena & Hnik 1963), 32 (Guttman & Hanzlikova 1966), 30 (Close 1967) and 27-30 (Andrew & Part 1972).

2.3.3.6 The Innervation Ratio and Specific Tension of Motor Units and Muscle Fibres

The innervation ratio of each type of motor unit can be calculated as shown in table 2.5 for the whole muscles. The relative innervation ratio (RIR) of each type of motor unit is calculated from the proportions of fibre types and motor units. The innervation ratio (IR)

Innervation Ratio of Whole Muscle

muscle	av. muscle force (mN)	av. motor unit force (mN)	no. motor neurons $\frac{\text{av. muscle force}}{\text{av. mu force}}$	muscle fibres counted	abs. innervation ratio (IR) $\frac{\text{no. muscle fibres}}{\text{no. motor neurons}}$
Control LG	1650	40	41	9431	230
Reinn LG	830	22	37	6508	176
Cont. Soleus	291	12	24	2071	86
Reinn. Soleus	170	17	10	1654	165

Table 2.5 Innervation Ratio of Control and Reinnervated Muscles. The number of motor units (= no. of motor neurons) can be estimated by dividing the average muscle force by the average motor unit force. The absolute innervation ratio (IR) is the number of muscle fibres/the number of motor neurons innervating the muscle (Dum et al, 1982).

of each type of motor unit is then the product of its' relative innervation ratio (RIR) x the absolute innervation ratio (abs. IR) of the muscle. The specific tension (mN per fibre) of the muscle fibres of each type of motor unit has been calculated from the ratio of the number of fibres per motor unit (IR) and the average motor unit tension. (The intermediate FI motor units were grouped with the FR motor units for this calculation and compared with the f.o.g. fibres as explained earlier for comparison of proportions of fibre types and motor unit types). These values are shown in table 2.6.

Innervation ratios showing larger motor units in control LG than in control soleus are consistent with the relative motor unit forces in the two muscles. The innervation ratio of reinnervated soleus motor units approaches that of reinnervated LG and both ratios are smaller than those for motor units in control LG. The calculated innervation ratios for each type of motor unit suggest that after reinnervation the fast motor units in LG have fewer fibres and the slow motor units are larger than controls. In soleus both fast and slow motor units have more fibres after reinnervation.

The reduced average motor unit tension of FF (glycolytic) motor units in LG after reinnervation can be attributed to a considerably reduced innervation ratio for this type of motor unit combined with

Table 2.6 Innervation Ratios of Motor Unit Types and the Specific Tension per Fibre of The Types of Motor Units.

The number of motor units was estimated (table 2.5) by dividing the whole muscle force by the average motor unit force. The absolute innervation ratio (abs. IR) is the number of muscle fibres innervated by each motor neuron (Dum et al 1982). The relative innervation ratio (RIR) of each type of motor unit is calculated as the proprotion of fibres per motor unit (= no. of motor neurons) of the corresponding type. The innervation ratio (IR) of each type of motor unit is the relative innervation ratio of each type x the innervation ratio for the muscle. The specific tension of the fibres of each motor unit type is calculated by division of the average motor unit tension by the number of fibres per motor unit. The physiologically identified FI motor units were grouped with the FR motor units and compared with fog muscle fibres.

Table 2.6

Motor Unit (MU) Innervation Ratios and Muscle Fibre Specific Tension

		CONTROL LG				REINNERVATED LG		
		FF/fg	FR/fog (FI+FR)	S/s		FF/fg	FR/fog (FI+FR)	S/s
% motor units		40	43	17		35	37	28
% fibre types		37	53	10		28	38	33
RIR - relative innervation ratio ($\frac{\%\ \text{fibres}}{\%\ \text{motor neurons}}$)		0.925	1.23	0.59		0.80	1.03	1.18
IR - innervation ratio abs. IR (table 2.5) x RIR	(x230)	213	283	136	(x176)	164	202	208
av. MU force (mN)		66	34	11		33	22	19
specific tension of muscle fibres force (mN)/fibre		0.31	0.12	0.08		0.20	0.11	0.09

		CONTROL SOLEUS			REINNERVATED SOLEUS	
% motor units		18	82		33	33
% fibre types		20	80		33	67
RIR - relative innervation ratio ($\frac{\%\ \text{fibres}}{\%\ \text{motor neurons}}$)		1.11	0.98		1.0	2.03
IR - innervation ratio (abs. IR x RIR)	(x86)	95	84	(x165)	165	335
av. MU force (mN)		13	10		22	14
specific tension of muscle fibres force (mN)/fibre		0.14	0.12		0.14	.04

a lower specific tension for the muscle fibres. The specific tension of the fibres in the reinnervated LG FR and S motor units is little changed. Although fibres of the FR and S motor units in control soleus have higher specific tensions than fibres in the same type of motor unit in control LG and reinnervated FR fibres in soleus continue to have high specific tensions, the fibres in reinnervated soleus slow motor units have much reduced specific tension - lower even than those of control LG slow motor units. Low forces found for slow motor units in reinnervated soleus result from the lower specific tension of fibres.

Superficial examination of unfixed muscles indicate that all fibres of rat soleus cross the mid-line of the muscle (where sections were stained and fibre counts made). Figure 2.18 shows the superficial portion of the LG comprising 3/5 of the cross-section at the widest part of the muscle belly (where our sections were counted) is pale. An estimated 98% of the fibres in this part of the muscle cross the mid-line and were included in the fibres counted. The deeper 2/5, which contains all the slow fibres and 95% or more of the f.o.g. fibres, is red. The fibres in this compartment of the LG are shorter (6-7 mm) and run obliquely distally from dorsal to ventral and towards two 'half-septae' which extend i) from the proximal tendon to just below the mid-line of the muscle (13 mm) and ii) from just above

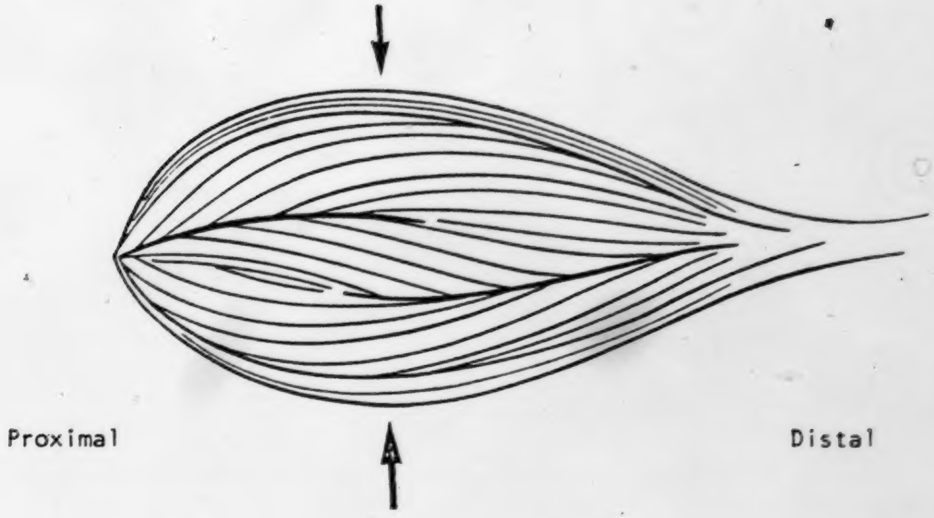


Figure 2.18 The ventral surface of the rat lateral gastrocnemius muscle showing the red, deep 2/5 which contains all of the slow and 95% of the f.o.g. fibres. Fibres in this portion of the muscle run obliquely as shown and are much shorter than the pale fibres. 98% of the fibres in the pale superficial part of the muscle cross the mid-line. Estimation of the proportion of fibres of the LG counted at the 'mid-line' of the muscle suggest that fibre numbers should be increased by not more than 20% and proportions of fibre types might be affected but the error would be no greater than 10%. A slightly greater correction would be needed for atrophic muscle owing to increased slant of the shorter fibres in atrophic muscle.

the mid-line of the muscle to the distal tendon (14 mm) in a muscle belly 24 mm long (figure 2.18). It can be estimated that 22% of the fibres in the red portion of the muscle were not counted at the mid-section. A calculation of the total proportion of fibres not crossing the mid-line would result in a correction of <20% in the observed muscle fibre counts and not more than 10% in the innervation ratios. These proportions might be increased in reinnervated muscle due to atrophy of the muscle having caused an increase the angle of the shorter oblique fibres with the long axis of the muscle.

Application of the calculation of innervation ratios to the soleus muscle motor units as shown in table 2.6 may be less valid owing to the small sample of motor units studied in both control and reinnervated muscle. The numbers caculated can nevertheless provide an indication that similar relationships can be shown between motor unit types. The result of reinnervation in soleus corresponds with that in LG in many respects. Motor units in control soleus have considerably fewer fibres than corresponding control LG motor units. After reinnervation the slow motor units in soleus have 4x the number of fibres of control soleus slow motor units and more fibres than are in reinnervated LG slow motor units while the fast motor units have normal numbers of fibres. This is in agreement with the deduction made in the preceding section that fewer nerve fibres reach soleus muscle, and that the slow fibres that do reinnervate soleus form large motor units in agreement with the finding by Lewis et al (1982) of very large slow motor units in reinnervated cat muscles.

2.3.4 Discussion

It is clear from these results that the metabolic pathway preferentially used by a muscle and its enzyme profile are partially resistant to the trophic control of the motor neuron. The good correlation between the histochemical profile and the physiological properties of the motor units which has been found for normal muscle (Burke et al 1973) has been disrupted in the reinnervated soleus muscle. Although the fibres of the originally fast LG muscle have altered their enzymic characteristics in such a way that motor unit and histochemical properties remain well correlated after reinnervation, the fibres of the originally slow soleus muscle demonstrate a high degree of resistance to alteration of their normally oxidative profile and of the acid-stability of their myosin ATPase enzymes even when reinnervated by a fast motor nerve. Although reinnervated soleus has a shorter contraction time than control soleus, it continues to be primarily an oxidative, fatigue-resistant muscle which has a higher proportion of oxidative fibres with histochemically acid-stable slow myosin ATPase than predicted by its small proportion (31%) of slow motor units.

This is in agreement with recent reports of a large percentage change in the contractile speed of cross-innervated cat soleus muscle accompanied by a much smaller percentage change in the histochemical fibre types of the muscle (Burke 1980; Edgerton et al 1980; Bagust et al 1981; Gauthier et al 1983). Other reports of asymmetrical effects of cross-reinnervation on fast and slow muscle also confirm the retention by the slow soleus muscle of an oxidative profile (Prewitt & Salafsky 1970; Sreter et al 1975).

Altered innervation of muscle causes synthesis of an altered myosin isoenzyme (Hoh et al 1980; Gauthier et al 1983), but the myosin is not completely re-specified. Cross-reinnervation of cat soleus by the nerve of a fast muscle was not able to suppress synthesis of a slow myosin light chain even after 15 months. The production of an altered hybrid myosin isoenzyme with different heavy chains (Gauthier et al 1983) in rat soleus under the influence of an increased proportion of fast motor neurons could explain the change in the contractile properties of the reinnervated muscle. Failure of the nerve to suppress the production of slow myosin light chains could explain the discrepancy between motor unit properties and histochemical profile. It can be concluded that conversion or partial conversion of slow muscle under the influence of its motor neurons can result in muscle with shorter contraction time having a faster myosin ATPase which has a mixture of heavy and light chain components but continues to react histochemically as slow, acid-stable myosin ATPase.

There is a tendency for the motor units to have size relationships similar to control muscle in both fast and slow muscles after reinnervation when force is used as the criterion of size, regardless of which target muscle has been reinnervated by the fast and slow nerve axons and regardless of the former type of the muscle fibre as described for retention of normal contractile speeds in the previous chapter. Relationships between size (force), contraction time and resistance to fatigue of motor unit types can still be demonstrated after reinnervation for the majority of motor units. The force and fatigue resistance of motor units as well as the contractile properties are related to the type of neuron innervating the muscle fibre.

Although reinnervated motor units show a much smaller range of forces the relationship between size as indicated by tetanic tension and contraction time (figure 2.12) is significant at the $p < 0.05$ level. The calculations of tables 2.5 and 2.6 indicate that there is a gradation in the number of fibres per motor unit in control muscle which agrees with the gradation of motor unit forces. In control muscle the fastest motor units tend to have the largest number of fibres as well as those with the greatest force output per fibre. After reinnervation all motor units in LG and fast motor units in soleus have similar numbers of fibres to each other while slow motor units have greatly increased numbers of fibres in both muscles. Oxidative fibres in control soleus (f.o.g. and s.) have greater specific tensions than those in LG. This remains true for soleus fibres reinnervated by fast nerve (f.o.g.) but not for soleus fibres reinnervated by slow nerve (s.). Fast fatiguable fibres in the fast LG and slow fibres in slow soleus are found to have greatly reduced specific tensions after reinnervation.

Classification of motor units by means of the "sag" criterion resulted in a "less powerful fraction of slow units relative to the number and size of the histochemically slow fibres... and led to the conclusion that slow fibres were inherently weaker than fast fibres" (Kernell et al 1983). Although the figures cannot be directly compared since other reports estimate specific tension as kg/cm² (Dum et al 1982; Kernell et al 1983) the method of classification used here also shows slow fibres to be less powerful than fast fibres. Each type of soleus fibre has greater specific tension than the corresponding type of LG fibre. While the motor neuron seems able to influence the force output of muscle fibres as well as contractile speeds, intrinsic muscle properties also appear to influence force ouput.

The size of the sample of motor units limits the confidence that can be placed in the interpretation of the data for soleus muscle. However, each motor unit property examined in the sample of motor units studied in control soleus and in the motor units in both muscles after reinnervation fits into the scheme of classification found for motor units in control LG (109 motor units). Classification by this method has produced results that agree with classification as established by previous research on muscle motor unit populations (Burke et al 1973; Kugelberg 1973; Chan et al 1982; Dum et al 1982) and therefore lends considerable credence to the results.

The loss of 29% of the original nerve fibres and of 20-30% of the muscle fibres and the continuing atrophy of muscle fibres indicate either that some nerve axons failed to reach or failed to make successful contact with denervated muscle fibres or that there has been some form of inappropriate matching of nerve to muscle and subsequent withdrawal of synapses. In either case the fast motor nerves did not innervate a greater than normal proportion of muscle fibres even in the presence of denervated muscle fibres. Guttman & Hanzlikova (1966) reported a loss of one third of the muscle fibres and 46% of the nerve fibres in reinnervated rat soleus and Bagust et al (1981) reported a similar loss of nerve fibres without increased motor unit size. Our results indicate an ability for slow motor units to increase in size - both in force and in numbers of fibres - in both the fast and slow muscle when reinnervated and tend to agree with the report of Lewis et al (1982) of very large slow motor units in reinnervated cat soleus.

These results also suggest that many of the results of previous experiments in which incomplete conversion of whole muscle properties after cross-innervation was found (Sreter et al 1975; Bagust et al 1981) may be explained in part on the basis of a mixed population of motor units in reinnervated muscle and in part by a limitation in the trophic influence of the nerve in transformation of muscle fibre properties.

Conclusion

In summary, it appears that many of the contractile properties of muscle fibres continued to be associated with the trophic influence of the motor neuron and the changes of myosin isoenzymes induced by altered innervation (Sreter et al 1975; Hoh et al 1980; Gauthier et al 1983). On the other hand, the oxidative properties of soleus muscle fibres appear to be intrinsic properties that are resistant to neurotrophic control.

Reinnervation of muscle by fast and slow motor neurons has been demonstrated to be non-selective. The proportion of slow motor units in both reinnervated muscles was found to be the same as the proportion of slow fibres in the nerve. Although there was a proportionately greater loss of slow nerve fibres after reinnervation, those fibres that survived were able to reinnervate much larger motor units than in control muscle. Muscle fibre specific tension was reduced after reinnervation in the fastest fibres of the fast muscle and the slow fibres of the slow muscle.

The dorso-ventral "bands" of type-grouped LG muscle fibres give the appearance of a strong role for contact guidance in the regrowth of the axons of the cut nerve, as does the regrowth of the nerve to soleus through its former perineurium. It is likely that the nerve re-entered the LG muscle by entering its original connective tissue sheath at the muscle surface. The connective tissues of both the nerve and muscle appear to have played an important role in directing reinnervation by providing "contact guidance" for the re-growth of the nerve.

The striking compartmentalization of each type of motor unit and the reduced force of fast motor units in reinnervated muscle will impair the fine control of movement. The phasic fibres needed for quick large-scale movements are clearly no longer located appropriately in the superficial part of the muscle in LG, and some former LG motor neurons now innervate soleus muscle, raising the question of whether the effects of reinnervation will be more maladaptive in the LG muscle. The fact that the postural soleus muscle has retained its oxidative metabolism may allow this muscle to continue to perform its original function with less disruption of normal function.

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3. APPENDIX I

The two extremes of circularity are shown in Figure 1.12 A and B (page 43). The notation is the same as indicated in the Methods, with the addition of the variable x to represent the length of axon which has become flattened. In the extreme case of Figure 1.12 B, where the axon has disappeared completely, x represents the length of the slit which remains. A number of parameters which are defined in the Methods and used throughout the Results can be readily calculated for the two extremes A) and B) and are listed in Table 2.7. We assume that the unflattened portions remain circular so that the myelin thickness is the same at all points.

Note that the axon perimeter will be the same in A) and B) if $x = \pi d/2$. The external perimeter will also remain the same since

$$2(x + \pi m) = \pi(d + 2m) = \pi D \qquad (1)$$

The length of all layers of myelin intermediate between the internal and external perimeter can also be shown to remain unchanged. Finally, the myelin thickness can be obtained in both extremes from the formula

$$m = (S - s)/(2\pi) \qquad (2)$$

To see how an axon could atrophy from A) to B) consider the intermediate example C) which again retains a constant thickness of myelin. The variable y represents the short axis of the axon. The corresponding values of the parameters are also listed in Table 1 for the intermediate case C). The axon perimeter will always be the same as that of the original circle if

$$y = d - 2x/\pi \qquad 0 \leq y \leq d. \qquad (3)$$

The two extremes are A) $y = d$ and $x = 0$, which corresponds to the circle and B) $y = 0$ and $x = \pi d/2$, which corresponds to the completely atrophied axon.

Substituting condition 3) into the formulae in the Table under column C) gives the simplified expressions of column D). Thus, by using this particular geometric transformation an axon could atrophy from a circle to a slit, while the myelin thickness m, the inner and outer perimeters s and S, and the ratio $g = s/S$ remain unchanged, as found experimentally. Note that the ratio of equivalent diameters d_{eq}/D_{eq} will not remain constant, but will decline to 0. It is this ratio which corresponds to the slopes calculated in Figure 1.5.

The particular geometric transform in Figure 1.12, in which m, s, S and g remain constant during atrophy is in fact one of a large class of such transforms. Any arbitrarily shaped axon can be approximated by a polygon, the level of approximation increasing with the number of sides of the polygon. If there is an n-sided polygon as

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[*] This theorem was prepared and written by Dr. R. B. Stein to support the experimental findings in this research and is included here with his permission.

	A)	B)	C)	D)
a = axon perimeter	πd	$2x$	$\pi y + 2x$	πd
S = external perimeter	πD	$2(x + \pi m)$	$\pi(y + 2m) + 2x$	πD
$\rho = a/S$	d/D	$x/(x + \pi m)$	$\frac{\pi y + 2x}{\pi(y + 2m) + 2x}$	d/D
α = axon area	$\frac{1}{4}\pi d^2$	0	$\frac{1}{4}\pi y^2 + yx$	$\frac{1}{4}\pi d^2 - x^2/\pi$
A = total fibre area	$\frac{1}{4}\pi D^2$	$\pi m^2 + 2mx$	$\frac{1}{4}\pi(y + 2m)^2 + (y + 2m)x$	$\frac{1}{4}\pi D^2 - x^2/\pi$
$\phi = 4\pi\alpha/a^2$ = axon circularity	1	0	$\frac{\pi(\pi y^2 + 4yx)}{(\pi y + 2x)^2}$	$1 - (\frac{2x}{\pi d})^2$
$d_{eq}/D_{eq} = \sqrt{\alpha/A}$ = ratio of equivalent axon and outer diameters	d/D	0	$\sqrt{\frac{\pi y^2 + 4yx}{\pi(y + 2m)^2 + 4(y + 2m)x}}$	$\sqrt{\frac{\pi^2 d^2 - 4x^2}{\pi^2 D^2 - 4x^2}}$

Table 1.1 Computed parameters for the simple geometric shapes of Fig. 1.12. The columns A), B) and C) correspond to the parts of Fig. 1.12 while the column D) corresponds to the simplifying assumption given by equation 3), which corresponds to a particular mode of atrophy for the axon.

shown in Figure 1.12D (page 43) with angles β_1, β_2, ..., β_n and sides D_1, D_2 ....., D_n, then we can construct another figure a distance m from the original polygon by drawing parallel lines of length D_1, D_2, ... D_n a distance m outside the original lines and connecting them with arcs (subtending angles γ_1, γ_2, ..., γ_n from circles of radius m. The perimeter of the inner figure is then

$$s = D_1 + D_2 + \dots + D_n \tag{4}$$

and the perimeter of the outer figure is

$$S = D_1 + D_2 + \dots + D_n + m(\gamma_1 + \gamma_2 + \dots + \gamma_n) \tag{5}$$

For an n-sided polygon

$$\beta_1 + \beta_2 + \dots + \beta_n = (n - 2)\pi \tag{6}$$

Also,

$$\gamma_n = \pi - \beta_n \quad , \text{ if } \beta_n \le \pi. \tag{7}$$

Thus, if all the angles are less than π,

$$\gamma_1 + \gamma_2 + \dots + \gamma_n = n\pi - \beta_1 - \beta_2 - \dots - \beta_n = 2\pi \tag{8}$$

Thus, $S = s + 2\ m$. So long as the inner layer of myelin remains the same length and the number of layers remain constant (which determines m), the outer perimeter will remain unchanged as will the ratio $g = s/S$. This general result is also the rationale for calculating the average thickness of the myelin from the formula given in the Methods. However, if one or more of the angles is greater than π, the simple relations derived above break down.

For example, consider the pentagon of Figure 1.12E in which one angle β_4 is greater than π. We have drawn a symmetric pentagon in which two angles β_1 and $\beta_2 = \pi/2$ and $\beta_3 = \beta_5$, but the same results will apply qualitatively to more general shapes. Use of a symmetric shape merely simplifies the calculations. For this pentagon

$\Sigma' \beta = 3\pi$, $\beta_1 = \beta_2 = \pi/2$ and $\beta_3 = \beta_5$, so $2\beta_3 = 2\pi - \beta_4$. Then,

$$s = \Sigma' b \tag{9}$$

and

$$S = \Sigma' b - 2\ell + m(\gamma_1 + \gamma_2 + \gamma_3 + \gamma_5) \tag{10}$$

Since $\gamma_1 = \gamma_2 = \pi/2$ and $\gamma_3 = \pi - \beta_3 = \gamma_5$

$$S = s + 3\pi m - 2\ell - 2\beta_3 m \tag{11}$$

However, $\tan\lambda = \ell/m$ and $\lambda = \pi/2 - \beta_3 = (\beta_4 - \pi)/2$

Substituting these expressions and simplifying,

$$S = s + 2\pi m - 2m(\tan\lambda - \lambda) \tag{12}$$

where $\tan\lambda \sim \lambda + \lambda^3/3$, for small values of λ Thus,

$$S \sim s + 2\pi m - 2m\lambda^3/3 \tag{13}$$

In the example shown where $\beta_4 < 3\pi/2$, $\lambda < \pi/4$ and $(\tan\lambda) - \lambda < 0.215$

$$s + 2\pi m(0.93) < S < s + 2\pi m \tag{14}$$

Thus, the difference is only a few percent, but as the myelin becomes more infolded, the difference will increase and the outer layers will either have to shrink relative to the inner ones or separate from them.

It is not the infolding per se which leads to a breakdown of the relation $S = s + 2\ m$, but the oblique angle. If the myelin becomes wavy with segments composed of arcs of circles in which the inner and outer layers are always separated by a distance *m*, it can easily be shown that the relation between *S*, *s* and *m* will still hold.